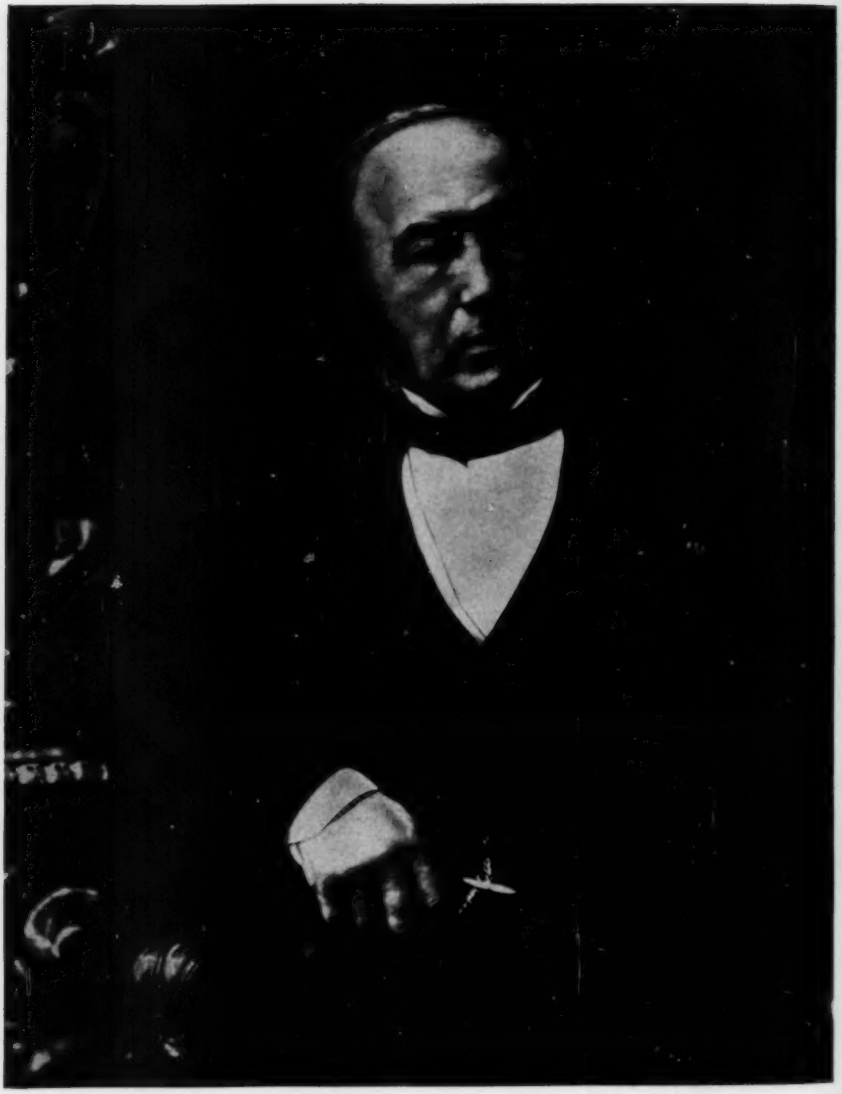
CLAUDE BERNARD

1813–1878



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July 12, 1813–February 10, 1878

The physician-in-chief of the Hotel-Dieu [1] looked first at the skillful and thorough dissection, then at the new intern whose name he could not recollect, and in his characteristically rude and abrupt manner he called out: "I say, you there, I take you as a demonstrator at the Collège de France." Thus, or so the story goes, began the association of Magendie and Claude Bernard, of whom it was later to be said that "one was the founder of Physiology, the other Physiology itself."

Bernard was then 26, tall, thin, dark haired, a 5th-year medical student.[2] Until his internship had begun a few weeks before this meeting, he had been living in a garret in the old Quartier Latin. This one-room apartment, shared with a comrade, had been not only bedroom, study, and kitchen to them, but also a laboratory where he had performed his first experiments on dogs and cats. He had passed all his examinations regularly and had excelled in anatomy, but had not been overly popular with most of his other professors, probably because of the obvious skepticism with which he received their teachings. Yet it was this critical attitude which was to lead him to revolutionize biological knowledge and medical thought. According to Bernard himself, his skepticism toward traditional medicine had been engendered not so much by his study of Francis Bacon's "Novum Organum" as by his having had to prepare each week during two years of his adolescence, out of spoilt herbs and left-over drugs, a pharmaceutical syrup purporting to cure all ills.

[1] Paris' oldest hospital (founded in 650) situated next to Notre-Dame Cathedral.

[2] The French medical curriculum entails 7 years of hospital and course work, followed by a thesis. Honor students, or "interns," live in the hospital from the 4th or 5th year on.

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Bernard was born on July 12, 1813, in Saint Julien (Rhone), a little village typical of the old province of Beaujolais. Hidden among the trellises, it was and still is inhabited almost exclusively by proprietors of small vineyards, their helpers, and agricultural artisans. The nearest small town and market center, Villefranche, is about 5 miles away. The countryside is pleasant, with moderate slopes covered with vines, wooded valleys and brooks running gently from the hills toward the Saone River. To the east the horizon is bordered by the white summits of the Alps. Such a rural community, situated far from the main Paris-Lyons road, had hardly been touched by the powerful philosophical, political, and scientific currents which had shaken the larger French cities through the Revolution and Napoléon's reign. In a small farm of this quiet village, Bernard passed his childhood playing with the village children and helping with the wine making. (A few years later, in the neighboring province of Franche Comté, was to be born in similar humble and rustic circumstances the son of a tanner and wine grower who was to become his friend and equally glorious colleague, Louis Pasteur.) To this farm Bernard returned almost every summer until the end of his life, there to restore his strength and his peace of mind as he watched the grapes ripen.

Because young Claude was an unusually bright child, the curé took a special interest in him and taught him Latin. Later he was sent to the secondary school at Villefranche, and after completion of its limited curriculum went to Lyons, there to pursue his studies and obtain his baccalaureate. This completed, Bernard, who had developed literary aspirations, decided to stay in the large city and make a name for himself as a playwright. Possibilities of support from his family having run out, he had to look for a job and — fortunately for the development of his future career — was offered a position as a pharmacist's aide. This situation gave him his room and board but did not at first carry any wages. On the other hand, it left him enough leisure to spend his evenings at the theater and to work on his plays. When his

vaudeville comedy "La Rose du Rhone" was accepted and met with definite success on the boards, Bernard, still in his teens, decided to embark on a more ambitious dramatic venture and started forthwith an historical tragedy in 5 acts entitled "Arthur de Bretagne."

Meanwhile, during the daytime, Bernard worked conscientiously at the pharmacy. He quickly developed such manual dexterity and neatness of preparation and dispensation that he was granted a small but steady salary. During the two years which he spent in this occupation, he acquired not only a certain knowledge of chemistry but also his persistent interest in poisons which was later to manifest itself in his memorable experiments on the action of curare, strychnine, nicotine, and carbon monoxide and in his "Lessons on the Effects of Toxic Substances." Also, it was while concocting at the end of each week the ever-variable panacea that he conceived his profound contempt for the prevalent state of therapeutics and his distrust for uncontrolled experiments and results.

In 1834, at the age of 21, having completed "Arthur de Bretagne," Bernard went by coach to Paris to show his manuscript to Saint-Marc Girardin, Professor of Literature at the Sorbonne and famous dramatic critic. His hopes were high; but Girardin, while recognizing that the play (which was later published) demonstrated a certain amount of literary ability, wisely dissuaded Bernard from devoting himself exclusively to writing. He added, "You have studied pharmacy; why not study medicine? You will be assured of a livelihood and may still manage to save free time for yourself in which to write plays if you feel so inclined."

When at Magendie's unexpected invitation in 1839, Bernard started what was to be a life-long association with the Collège de France, the chairs of medicine and natural history had already for 300 years been graced by such professors as Vidus Vidius, Jacobus Sylvius, Riolan, Buffon, Daubenton, Cuvier and Laennec; the Collège now boasted not only Magendie but also Flourens, the discoverer of the role of the

periosteum and epiphyseal cartilages in the growth of bones. Yet material facilities for biological research and teaching were still almost nonexistent. The chemical and physiological laboratory consisted of one small room; only one or two assistants were available to provide technical help. This meant that helping the professor in the preparation of his experimental lessons was arduous and time-consuming. However, these routine contacts enabled Claude Bernard to receive lengthy practical demonstrations from Magendie almost daily. It is not necessary to remind the readers of the Journal of Nutrition of the achievements of Magendie; the excellent study by Paul F. Fenton recently published in these pages vividly brings back to life the powerful figure of the man who, according to Michael Foster, "did alone with his own hand in France as much as, or more than, was being done by others in all other lands." But it is worth noting that Bernard found him, in spite of his somewhat difficult character, a useful teacher. Magendie, whose dislike of all theoretical discussions led him to call himself "a mere scavenger of facts," furnished a striking contrast to the vitalist doctrinaires of the Faculté de Médecine. His exalting of experimentation found at first a receptive ear in the skeptical Bernard but the genius of the young man was soon to outgrow even the lessons of his illustrious master. Beginning with his very first original labors, he put experimentation in its right place in biological research, between the careful elaboration of a logical hypothesis and the subsequent rejection or further development of the original theory.

Bernard completed his 7-year medical curriculum in 1841 and from then on, as chief assistant to Magendie, was able to devote almost all of his time to research. In May, 1843, he published his first communication, dealing with the role of the chorda tympani in taste and in hearing and with the relation of lesions of this nerve to facial paralysis. (He was to come back again and again to this subject of study and to derive, from consideration of the chorda tympani, some of his greatest advances in physiology.) In December of the same

year he completed his thesis for the Doctorate of Medicine, entitled: "On Gastric Juice and its Role in Nutrition," which marked the beginning of the most famous series of physiological investigations ever conducted. The central point of the thesis was his demonstration that cane sugar, if injected directly into the veins, is quantitatively excreted in the urine, while this does not occur if the sugar has been previously incubated with gastric juice at body temperature. From this he concluded that cane sugar as such was not a proper nutrient for the tissues, but that the effect of digestion was to transform it into utilizable simpler substances. The thesis also included the demonstration, through the ingenious use of simultaneous injections of potassium ferrocyanide and ferrous sulphate, of the fact that the acid of gastric juice makes its appearance on the surface and not in the depths of the gastric glands. His next study resulted in a memoir on the chemistry of certain pigments found in the human body. It was followed by his second important discovery, brought about by his observation, *in vivo,* of differences in the site of beginning digestion and absorption of fat in the intestine of rabbits, on the one hand, and of dogs on the other. Correlating this with the differences in anatomical position of the opening into the intestine of the pancreatic duct, he was thus to start his remarkable researches on the function of the pancreas, and to throw light on the hitherto unknown processes of intestinal digestion.

Since the work of Johannes Müller and of Beaumont, the changes undergone by the food in the stomach had been considered as identical with digestion as a whole. In a series of memoirs presented to the Société de Biologie and to the Académie des Sciences, Bernard described in rapid succession the several distinct actions of pancreatic juice, having made use of the pancreatic fistula for the first time and having devised new chemical methods. First he analyzed the action of pancreatic juice on fats, demonstrating that, after emulsifying them, it split them into fatty acids and glycerine. Second, he showed that pancreatic juice acted on starch to

transform it into sugar. And, finally, he elucidated the action of pancreatic juice on proteins; pointing out that bile precipitated the proteid products of gastric digestion and stopped peptic changes, he demonstrated that the pancreatic juice further acted on these precipitated nitrogenous matters as well as on the proteids which had escaped peptic action. Thus "gastric digestion is only a preparatory act." When, as a result of his studies on the pancreas, the Académie des Sciences awarded him in 1850 the prize in experimental physiology, it only sanctioned the already well-publicized fact that there now were two great physiologists in Magendie's department. Incidentally, it is worth noting that his own research on digestion and nutrition still left Bernard enough freedom to conduct simultaneous researches on the function of the spinal nerves, work which was allied to Magendie's interest. In many cases he was able to define for this inspired but often careless investigator the precise conditions in which experiments should be performed. He was thus able, repeatedly, to rescue his professor from needless controversies by neatly confirming the validity of important conclusions.

Bernard's next great discovery, that of glycogen and of its role in the economy of the human body, presents an odd and almost unique phenomenon. It is almost impossible for us, a century later, to realize the revolution which it brought to the entire scientific outlook of his contemporaries. On the other hand, only a few of them were able to comprehend the unlimited perspectives which Bernard was then opening to biologists.

The contemporary implications of the discovery of glycogen may be better understood if it is recalled that at the time Bernard first started his work at the Collège de France the views expounded by the great chemists, Dumas and Boussingault, in their "Essays on the Chemical State of Organisms" dominated all biochemical thought. Briefly stated, their theory rested on the supposedly sharp contrast between the metabolism of plants and animals. The metabolism of plants was regarded as essentially synthetic, constructing from

inorganic materials the complex organic compounds, carbohydrates, proteins and fats which constitute the chemical basis of life. Animal metabolism was thought to be incapable of any syntheses, making use only of the ready-made components and deriving the energy necessary for growth and the maintenance of life from their resolution into their inorganic constituents. Bernard wanted to follow the products of intestinal digestion and decided to start with the organic cycle of sugars, hoping by this approach to understand the etiology of diabetes. At his first step he demolished the almost universally accepted theory of Dumas.

Turning from his eradication of past errors to future developments, it is no exaggeration to say that the new concepts elaborated by Claude Bernard — internal environment, functional interdependence of tissues, continuous synthesis and degradation, and homeostatic neuro-humoral regulations insuring the "constancy of the milieu intérieur" — all originated in his thorough and harmonious inquiry into the fate of absorbed dextrose.

The discovery of the glycogenic function of the liver was thus not a matter of chance. Bernard, always a methodical investigator, decided to study sugar metabolism by following dextrose along the physiological pathway: passage from the intestine into the portal vein, through the liver, from there to the right heart, the lungs, the left heart, and thence to the tissues. He proposed further to see whether, by interfering with utilization at one or the other of these stations, he could bring about diabetes. He achieved both these ends. He was equipped for this search with the method which he and his friend, the chemist Barreswil, had developed to determine glucose, using the reduction of cupric sulphate. The reduction of cupric salts has remained to this day a basis of glucose determinations.

In his first experiment Bernard demonstrated the presence of an abundant amount of sugar in both the portal and the hepatic veins of a dog fed a diet rich in starch and cane sugar. On the face of it, the liver seemed not to utilize the

absorbed sugar. However, Bernard's critical mind questioned his own assumption of the identity of the portal and of the hepatic sugar. Repeating his experiment with another dog fed only meat, he demonstrated that sugar was present neither in the alimentary canal nor in the portal vein, but that the blood in the hepatic vein was loaded with dextrose. "The liver produced sugar out of something which was not sugar; and within it lies the secret of diabetes." To confirm this result, Bernard repeated his experiment in every possible way; on starved dogs, and on dogs fed meat alone for a long period. To eliminate possible errors of localization, he ligated the mesenteric veins, the veins from the pancreas, the splenic vein, and the vena portae near the liver. He confirmed his identification of the sugar produced by the liver by chemical and physical methods, as well as by fermentation tests, producing alcohol from it. In 1849 he published the first account of his research on carbohydrate metabolism in a memoir addressed to the Société de Biologie; in 1850 a fuller account was published in the *Comptes Rendus* of the Académie des Sciences. Continuing his researches in this field, he studied the rate, under different conditions, of what he called the "internal secretion" of the liver. He showed that this hepatic sugar, though not coming directly from the food, was influenced as regards its quantity by the state of nutrition and by the nature of the food. It was diminished by starvation and markedly increased by proteins, gelatin in particular, or by starches. It was hardly, if at all, increased by fatty food. This, as well as the influence of other factors such as exercise and age, he expounded in his thesis for the degree of Doctor of Science, which he received in 1853.

Bernard, incidentally, had not forgotten his ambition of provoking diabetes experimentally, which he considered to be the first step toward the eventual development of a rational therapeutic approach. In 1849 he communicated to the Société de Biologie the fact that he had succeeded in making rabbits and dogs diabetic by puncture of the 4th ventricle. He had been led to this result by a methodical search based on the

assumption that the secretion of the liver, like that of many other glands, must be influenced by an appropriate nerve. Remembering some older experiments of his on the trigeminal nerve in which he had produced secretion of tears and saliva by puncturing the nerve at its origin in the brain, he conceived the idea of applying the same method with respect to the vagus at its origin in the floor of the 4th ventricle. After he had produced the lesion, sugar poured from the liver into the blood and spilled over into the urine. While Bernard himself later demonstrated that the vagus was not the direct channel of the excitation caused by puncture of the 4th ventricle, he had by his systematic investigation, and guided as ever by a rational theory, arrived at his goal of producing temporary diabetes. He further showed that section of the cerebellar peduncles led to the appearance in the urine of albumin and sugar.

Bernard proceeded with his systematic analysis. He removed a fresh liver from the body of a dog and perfused it until the water issuing from the hepatic vein contained no sugar; a sample of the liver so washed was then also free of chemically detectable sugar. Bernard maintained the washed-out liver at body temperature for a few hours and then perfused it again. This time the water issuing out of the hepatic vein was loaded with dextrose. Bernard interpreted this result as demonstrating the presence of a "glycogenic substance" in liver.

The almost incredible sequence of experiments which followed this deduction illustrates Bernard's dominant role in the genesis of modern investigative biochemical methods. First he strained the liver free of blood vessels and connective tissue; then, by means of alcohol precipitation and subsequent washing with ether, he was able to prepare an impure dry preparation of glycogenic substance, convertible to dextrose by fermentation. Seeking next to perfect his technique of preparation, he developed the potash-alcohol procedure which is still used to prepare glycogen. Having thus obtained a pure product, he was able to conduct, with Pelouze, an

elementary analysis and to demonstrate that the empirical formula obtained verified his hypothesis as to the carbohydrate nature of glycogen. What is more, he showed that a decoction of fresh liver could convert glycogen into dextrose; if this decoction was previously boiled, no such transformation took place. And Bernard concluded that the liver contained ferments, probably of a proteid nature because of their heat lability, which normally convert glycogen into dextrose, this transformation preceding the further degradation of dextrose by other ferments in tissues. He further compared the transformation of glycogen to dextrose by the liver ferments to that of starch to dextrose by the ferments of the germinating seed, asserting the analogous nature of starch and glycogen and the probable similarity of the two transformations.

By virtue of his discovery of the color reaction of iodine and glycogen, he was able for the first time to study physiological phenomena by histochemical methods. For instance, he followed the variations in liver glycogen in the embryo at the different stages of intra-uterine life (from conception to parturition. He examined the displacement of the equilibrium between synthesis of glycogen from dextrose and degradation of glycogen to dextrose. Bernard was always careful to publish all these results as soon as he had confirmed them to his own satisfaction. Yet at no time did any of his intensely interested contemporaries succeed in anticipating any of the carefully planned steps of this study. As a consequence of this work, the conventional concepts of physiology and biochemistry, vitalism, Dumas' contrast between chemical processes in plants and animals, Bichat's functional separation of the different tissues, all were shattered. The foundations of present-day physiological chemistry, endocrinology, enzymology and chemical embryology had been solidly established.

It is proof of Bernard's unequalled genius that during this same period he accomplished in still another field of physiology an achievement which alone would have assured

him of a place among the greatest physiologists of all time, namely, his discovery of the vasomotor mechanism in 1851. While studying the influence of the nervous system on heat production in the body, he showed that divisions of the cervical sympathetics on one side produced an elevation of temperature on that side. He also observed an even more marked rise following removal of the superior cervical ganglion, and found that sensitivity was also increased on the same side. Both of these phenomena he interpreted by postulating concomitant vascular effects. Testing this concept, he was able, by galvanic stimulation of the distal portion of the divided sympathetic, to produce diametrically opposite results, namely, a fall of temperature associated with intense vaso constriction. These findings were soon confirmed by Bernard's friend, Brown-Séquard, and had an immediate impact on contemporary physiologists and physicians. Yet it was again Bernard who best understood and exploited the implications of his discovery. From the outset he had pointed out that, through the action of the vasomotor system on the arterioles, the nervous system could in fact modify the chemical activity of the tissues. An increase in the supply of oxygen and nutrients enhanced tissue activity, while a decrease, brought about by vaso-constriction, depressed it. The intensity of the metabolism of an organ could thus be brought into conformity with the changing requirements of the organism.

Bernard completed his work on the vasomotor system by analyzing the different effects of the sympathetic and the parasympathetic systems. In 1858, after a methodical study of many exocrine organs, he observed that when a gland is actively secreting, the blood which drains from it remains red and arterial in nature. For instance, he showed that when the chorda tympani nerve was stimulated, the venous blood issuing from the submaxillary gland was bright red. If, on the other hand, the sympathetic nerves supplying the gland were stimulated, the blood turned dark red. A similar antagonism could be demonstrated in other glands. Accordingly, Bernard concluded that the chorda tympani and allied nerves were

vasodilators and capable of accelerating the blood flow to such an extent that the blood did not lose its arterial color in passing through the parenchymal capillaries. Conversely, the sympathetic nerves slowed down the blood flow and permitted the gaseous exchange to proceed to completion. By this work the fundamental facts of vasomotor physiology were established and a rational approach to the therapy of cardiovascular disorders became possible. When in 1847 Bernard gave his first lecture as deputy for the increasingly infirm Magendie, he started with these words: "Scientific medicine, gentlemen, which it ought to be my duty to teach here, does not exist." A few years after this statement was made he had given many essential aspects of medicine a rational basis.

Recognition, often a slow process in France, came relatively quickly in the case of Bernard. In 1854 the new chair of general physiology was created for him at the Sorbonne. (He kept this appointment until 1868, when he relinquished it to his pupil, Paul Bert.) The same year he was also elected to the Académie des Sciences to replace the surgeon Roux. In 1855, at the death of Magendie, Bernard was elected by the professors of the Collège de France to replace the old master who, 15 years earlier, a few weeks after Bernard had started his work in the laboratory, had suddenly told the young medical student in his customary gruff way: "You are a better man than I am." (Later in 1868 Bernard was also to replace Flourens as a member of the Académie Française and, in 1869, he was appointed a Senator.)

It is not possible to discuss here in detail Claude Bernard's other major contributions to science. But some important results of his investigations should be enumerated. As early as 1846 Bernard had already demonstrated the inhibitory effects of the vagus on the heart. He followed this observation with several studies on the general subject of inhibition. The study of poisons, which had fascinated him since the early days of the Lyons pharmacy, again aroused his interest, and from 1850 to 1852 he established the action of curare, nicotine, and other drugs on the nervous system, in particular demon-

strating the differential effects of curare on motor and sensory fibers. Perhaps even more remarkable was his analysis of the physiological action of carbon monoxide. In 1848, Bernard observed the peculiar color of the blood in carbon monoxide poisoning. By 1855 he had arrived at the conclusion that the red corpuscles were concerned in the retention both of oxygen and of carbon monoxide in the blood, and confirmed it by his demonstration of the beneficial effects of blood transfusion in carbon monoxide poisoning. "I was thus led," says Bernard, "to find that this gas (carbon monoxide) rapidly poisons animals, because it instantly displaces the oxygen of the red corpuscles and cannot itself be subsequently displaced by oxygen." In the course of this study, Bernard developed the fundamental method of determination of blood oxygen content. Among other notable achievements must be mentioned his demonstration in 1858 of the fact that the growths which readily appear in a solution containing gelatin and dextrose exposed to air fail to appear if the solution, previously boiled, is supplied exclusively with air which has passed through a red-hot tube. He interpreted this correctly when he concluded that the growths are therefore not spontaneous, but are due to airborne germs which are killed by passage through the red-hot tube. Pasteur later repeated and extended this experiment in his classic series of investigations refuting the spontaneous generation theory, and Bernard, both in private and at the public sessions of the Société de Biologie, the Académie de Médecine and the Académie des Sciences, always encouraged and supported him. (His only disagreement with Pasteur came much later, when Bernard argued, correctly, that the activity of ferments need not be dependent on the presence of living cells. Bernard's death interrupted the publication of the full results of experiments, conducted at the Collège de France and in the old paternal home in Saint Julien, which effectively demonstrated, 20 years before Buchner, that sugar could be degraded to alcohol by ferments extracted from lysed yeast cells.)

The later years of Bernard's life — from the time of his appointment to the Sorbonne and the Collège de France — were marked by continuing discoveries. They were also years of considerable teaching activity. Lecturing did not come easily to Bernard but he considered it an important duty and almost every year published a volume of lessons which, taken together, constitute the major opus of physiological literature: Lessons on Experimental Physiology (1854–55, two volumes), Lessons on the Effects of Poisons and Drugs (1856–57), Lessons on the Physiology and Physiological Properties and Pathological Alterations of Fluids in the Organism (1859, two volumes), Lessons on Experimental Pathology (1860–64), Lessons on Tissues (1863), Lessons on Anaesthetics (1869), Lessons on Asphyxia (1870), Lessons on Animal Heat (1871), Lessons on Diabetes (1873), Lessons on the Phenomena of Life Common to Plants and Animals (1872–73), and lastly his Lessons on Operational Physiology (1878). To these must be added hundreds of original memoirs and review articles, and above all, his Introduction to the Study of Experimental Medicine (1865), the clearest exposition ever written of biological epistemology, and a masterpiece of scientific style.

But Bernard was more than a profound and encyclopedic lecturer and writer. He was an inspiring and beloved teacher. The grave and kind physiologist had many friends, of whom the closest were perhaps his colleagues of the Collège de France, Berthelot, famous both as the chemist who had first performed the "total synthesis" of innumerable organic compounds and as a lucid foreign minister and statesman, and Renan, the great historian and philosopher, author of "The Origins of Christianity." Bernard's numerous disciples formed a brilliant school of physiologists, and all had for him an admiration and affection which almost amounted to worship. Among his French pupils and "preparateurs" the closest to him were Paul Bert and Dastre, later professors at the Sorbonne; Richet and Ranvier, later professors at the Faculté de Médecine; and D'Arsonval, later professor at the

Collège de France. Kühne of Heidelberg, Tarchanoff, and Rosenthal were among his many foreign pupils and assistants. The veneration which he inspired is best exemplified by the dedication in the "Claude Bernard" of Sir Michael Foster:

"To the Physiologists of France, both to those who had the happiness to know Claude Bernard in the flesh and to those who, like myself, never saw his face, this little sketch is dedicated in the hope that, as he has been to me a father in our common science, so I may be allowed to look upon them as brethren."

When Bernard died, on February 10, 1878, all Paris wept. On the unanimous vote of the Chamber of Deputies, state funerals were conducted for him, the first man of science or letters to be so honored by the traditional ceremonial hitherto reserved for kings, presidents and commanders-in-chief. The illustrious and aged Dumas, to whom the world owed the first determination of atomic weights and the theory of chemical functions, but whose system of biological chemistry Bernard had once shattered, was the chief speaker. He extolled the man "whose religion was Truth." Bernard's memory was to remain extraordinarily present and vivid in all institutions of learning he had honored with his association.

It is difficult to analyze the main elements of Bernard's greatness. What may be called the contributory factors are easier to describe. Bernard combined with a capacity for hard and prolonged work in the laboratory, the apprecation of the importance of leisure spent in quiet meditation. His adherence to exact truth was absolute, and he was always ready to recognize the limitations or the error of what had seemed like a promising idea until tested in the laboratory. His technical skill was superb, both as an experimental surgeon and as a biochemist. Yet essential as these characteristics were, they are not sufficient to explain his unequalled series of fertile discoveries. His "Introduction to the Study of Experimental Medicine" as well as other material which

was to be included in his unfinished "Principles of Experimental Medicine" give us clues to a better understanding of his genius. Bernard, first of all, believed strongly in the necessity of always having a working hypothesis, derived from perusal of the literature and observation of natural phenomena, before starting on the experiment proper. He used to say: "He who does not know what he is looking for will not lay hold of what he has found when he gets it." In a few lines which time has abundantly confirmed, Bernard decries the sterility of experimental work begun without the benefit of a starting hypothesis, and where the investigator relies only on statistical consideration of accumulated results to discover a natural law. This practice, he pointed out, is in fact "a return to the empiricism of the Middle Ages." A lively but rational imagination would appear to constitute the first prerequisite to greatness in science, and this Bernard had to the highest extent. But imagination is not equivalent to genius unless it is joined to what was perhaps Bernard's outstanding characteristic, the ability to devise the single, definitive crucial experiment which will test a far-reaching hypothesis. Bernard, like a great general who maps and executes a campaign by striking at the vital points of the enemy hosts, and takes only those positions which have to be taken to bring decisive victory, never wasted any time on experiments which were not essential to his progress. And, of course, his skill allowed him to perform these experiments in a minimum of time and with a maximum of precision.

And finally, there is the matter of interpretation of experimental data. Bernard had an extraordinary capacity for extracting from his results the most general and far-reaching conclusions that could be solidly supported by them; hence, his role as a progenitor in so many branches of the biological sciences.

For all of us, students of the phenomena of life, humbly treading the paths which Claude Bernard opened, using the words he coined, the methods of thought he developed, he

has remained and will always remain "a father in our common science."

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Bibliographical sources include first the writings of Bernard himself, a list of which can be found in the biographical notice written by his pupil Dastre for Charles Richet's Dictionnaire de Physiologie (Alcan Editeur, Paris, 1897). In preparation of this little study constant use has also been made of the "Claude Bernard" of Sir Michael Foster (T. Fisher Unwin, London, 1899). The writer is deeply grateful to Dr. George R. Cowgill for kindly editing the manuscript. The constructive editorial criticism of Dr. Theodore B. Van Itallie, of the Nutrition Department, Harvard School of Public Health, and the Peter Bent Brigham Hospital is also gratefully acknowledged. Last but not least, the writer had the invaluable advantage of the advice and encouragement of Professor André Mayer of the Collège de France, the Académie des Sciences and the Académie de Médecine, pupil of Dastre, the pupil of Bernard.

RAW VERSUS PROCESSED CORN IN NIACIN-DEFICIENT DIETS

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TWO FIGURES

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Pellagra has been found to be associated generally with populations eating large quantities of corn ("maize," in the British literature) and little animal protein. Recent work (reviewed by Krehl in 1949), first with rats and then with other species, has confirmed previous observations that a high corn diet increases the dietary requirement for niacin.

In Mexico there is very little primary pellagra, although there is a high per capita consumption of corn by some sections of the population, which also eat only small quantities of "animal protein" foods. One difference, among others, between the Mexican conditions and those pertaining to pellagra areas is that the corn in Mexico is eaten in the form of "tortillas" prepared in the first stage by cooking the whole corn in lime water. The vitamin composition of this material has been studied by Cravioto et al. ('45). Henderson and his co-workers ('47) found that the inclusion of cornstarch, autoclaved in an alkaline solution, in a ration which otherwise produced niacin deficiency in the rat, prevented the deficiency from appearing. In view of these results, it was considered important to see if corn cooked in the Mexican manner was less pellagragenic for the rat than untreated corn meal.

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EXPERIMENTAL

Housing and feeding of rats

Twenty-one-day-old weanling rats bred from the Rowett stock colony of hooded Lister rats, which received a stock ration (Thomson, '36) unless the contrary is stated, were housed individually on wire screens and fed the experimental diets ad libitum for a further 21 or 28 days in experiment 1. Each experimental diet had the following percentage composition: casein (alcohol-extracted) 7, L-cystine 0.15, linseed oil 2, cod liver oil 1, corn meal preparation (see below) 42, sucrose plus minerals [2] plus vitamins [3] 47.85.

The 42 parts of corn meal were prepared as follows for the different diets:

1. *Lime-cooked corn.* Lots of 2,500 gm whole yellow corn, 60 gm calcium oxide and 6 l of water were heated in a bath to 80°C. for 40 to 50 minutes with occasional stirring and then left to stand overnight. This mixture had a pH of 11. Next morning the mixture was neutralized with approximately 20 ml syrupy phosphoric acid. The solids were dried in a hot air tunnel at a temperature kept below 80°C., and the liquor previously decanted off was gradually added back so that all the starting materials were present in the final dried product. This was then ground to a fine meal. It was calculated that 42 parts of this product would contain approximately two parts of added tri-calcium phoshate.

2. *Control corn.* Whole yellow corn, from the same batch referred to above, was ground to a fine meal, and to each 40 parts were added two parts of tri-calcium phosphate.

3. *Reconstituted corn mixture.* Samples were obtained from a plant carrying out the standard "wet-milling" process in which the cleaned corn is steeped in dilute sulfurous acid kept at 52°C. for 36 to 48 hours, before separation into starch,

[2] NaCl, 0.9; KCl, 0.5; NaH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.4; $FeSO_4.7H_2O$, 0.2; $MnSO_4.5H_2O$, 0.02; KI, 0.005 parts per 58 parts of basal.

[3] For each 58 gm of the basal mix: choline chloride, 0.20 gm; thiamine, pyridoxine, riboflavin, folacin and 2-methyl-1,4-naphthoquinone, each 0.3 mg; Ca-*d*-pantothenate, 2 mg; tocopherol acetate, 1.5 mg; biotin, 0.01 mg.

gluten, germ, and feed meal fractions (Jacobs, '44). An attempt at reconstitution of 42 parts of "control corn," as above, was made with the following mixture: starch 28 parts, germ 5 parts, corn feed meal 5.5 parts, corn gluten 1.5 parts, and tri-calcium phosphate two parts.

In addition two further preparations were made. In the first, the starch was cooked with calcium oxide as described above for whole corn. In the second the germ, gluten, and feed meal portions were lime-cooked before reconstitution, and the starch was left untreated.

In experiment 3, in which these preparations were fed, the "control corn" diet was made up with the batch of corn that was used in the milling plant to obtain the corn fractions.

Experiment 1

Procedure. Thirty-two weanling rats, from 4 litters, each cut down at birth to 4 males and 4 females, were distributed among 4 diets — "cooked" and "control" corn, each with and without a supplement of 3 mg niacin per 100 gm of diet. The diets were fed moistened with water.

Results. The results are shown diagramatically in figure 1. It may be seen that the rats receiving the lime-treated corn grew much better than those receiving the untreated corn. With the latter, the addition of niacin resulted in growth comparable to that obtained with the lime-treated corn. The analysis of variance technique confirmed that the interaction of "cooking" and "niacin" effects was highly significant ($P < 0.01$).

Experiment 2

Procedure. As many workers have shown that small details of technique can influence the production of niacin deficiency in rats, a second factorial experiment was set up using 12 litters, from each of which 4 sisters were used, and with the following variables:

a. "Control" vs. "cooked" corn (neither having a niacin supplement).

b. Feeding dry vs. wetting fresh feed daily.
c. Mother and litters with access to stock diet up to weaning vs. change-over of mother to "raw corn" diet when litters were 16 days old.

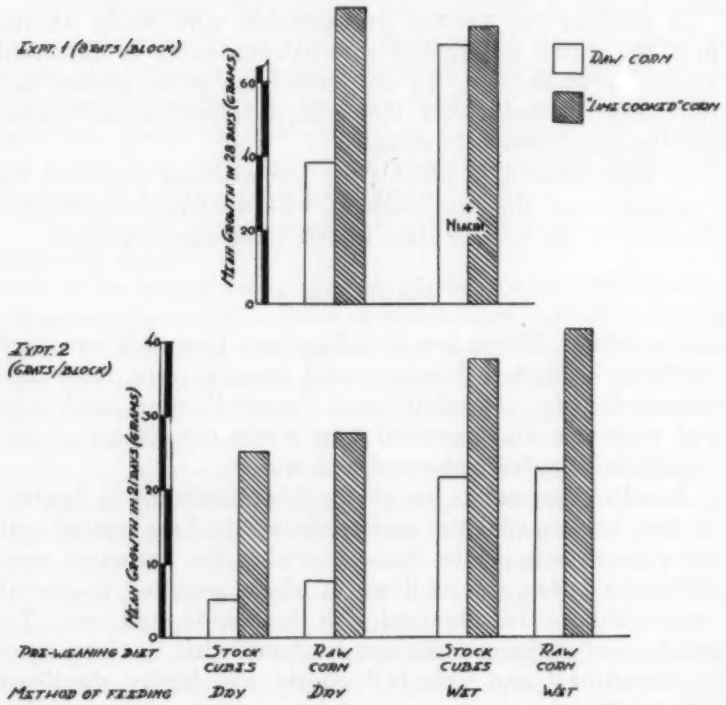

Fig. 1 Growth of rats fed raw and "lime-cooked" corn diets, with and without added niacin and under different conditions.

Results. The mean growth on the various diets is shown in figure 1. From the statistical analysis it was found that depriving young rats and their dams of access to stock cubes (a high-vitamin, high-tryptophan ration containing no corn products) in the last week of suckling had not affected their growth significantly on any diet. Wetting the diets had a

significant growth-promoting effect under all conditions ($P < 0.01$). Under each set of conditions, growth on the "lime-cooked corn" diet was superior to that on the "raw-corn" diet ($P < 0.01$).

Experiment 3

Procedure. Here it was hoped to test, first, whether a reconstitution of the fractions obtained from the commercial wet-milling process for corn would be found to have a "protective" effect similar to the apparent effect of lime-cooking and, second, if the processing had not had such an effect, the effects of lime-cooking separate fractions.

Seventy-two rats, consisting of 4 sisters from each of 18 litters, were distributed over 4 experimental diets. Of the 18 rats on each ration, 9 were given daily supplements of 0.5 mg niacin by mouth, and the remaining 9 received no supplement. All the diets were fed moistened with water. The diets were:

a. Raw corn.
b. Reconstituted corn.
c. Reconstituted corn with lime-cooked starch.
d. Reconstituted corn with lime-cooked germ, feed meal, and gluten.

Results. These are shown diagramatically in figure 2. Statistical analysis showed that:

a. The difference in growth rates between the groups fed the reconstituted corn diet with and without niacin was as great as that between the two groups fed the "control corn" rations with and without niacin.
b. Lime-cooking the starch had a significant growth-depressive effect on the rats fed niacin ($P < 0.05$) but no effect in the absence of added niacin.
c. Lime-cooking the gluten, germ and feed fractions did not change the growth obtained with niacin, and significantly increased the growth in the absence of niacin ($P < 0.02$).

DISCUSSION

The results of the three experiments seem to show that for the rat the method employed of cooking whole-corn kernels with lime water reduces the so-called "pellagragenic" effect of the corn.

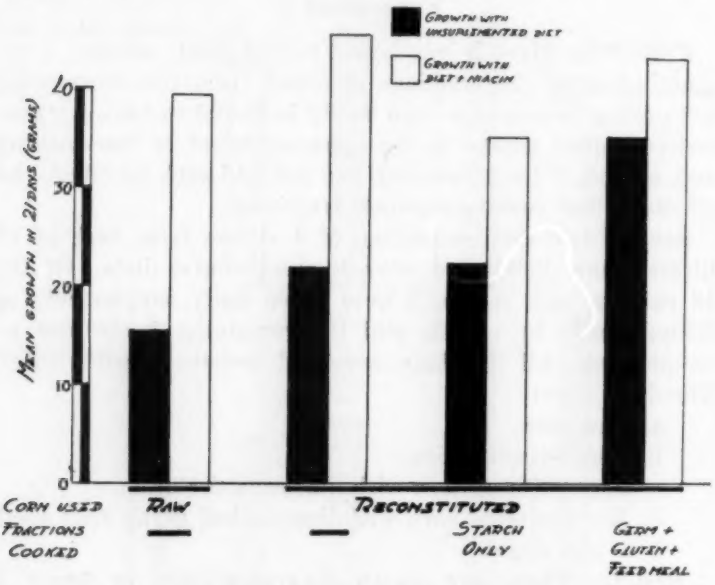

Fig. 2 Growth of rats fed diets with and without added niacin according to the treatment of the corn fractions.

On the other hand, the fairly severe treatment given to corn in the wet-milling process, which includes steeping for 48 hours in a weak sulfurous acid solution, does not have this effect, judging by the results in the third experiment.

The observation in experiment 2 that wetting the diets increases the growth rate of the rats even on the normally "pellagragenic" raw corn diet suggests caution in the interpretation of these findings.

Henderson and his co-workers ('47), who fed to rats a diet that required niacin supplementation for good growth, found

that the replacement of the raw starch in the diets of these animals with dextrinized starch eliminated the requirement for niacin. In their experiments the dextrinization was carried out by autoclaving the starch in a solution of sodium hydroxide.

In experiment 3, described above, it was hoped to show whether the lime-cooking effect could be explained by a similar change in the starchy portion of the corn during the treatment. This did not appear to be the case, and the full change seemed in fact to be produced by treating only the mainly non-starchy fractions — the gluten, feed meal, and germ.

It seems more likely that the present results can be explained by the findings of Kodicek (Chanduri and Kodicek, '50; Kodicek, '51), reported during the course of these experiments. He demonstrated the presence in corn and other cereals of a "precursor" of niacinamide which is normally unavailable to the rat. It is liberated by alkaline but not acid hydrolysis, as shown by biological assay with rats.

It is not suggested that the present findings explain the low incidence of pellagra among people eating lime-cooked corn, but they do appear to provide a starting-point for further work. It will be of interest to carry out similar studies with corn cooked by the procedures used in areas having a high incidence of pellagra. Until such experiments are carried through, it will not be possible to assess the possible practical significance for human nutrition of this method of cooking. Nevertheless, these studies emphasize again the importance to health of many of the cultural food patterns of man. Cooking corn in lime water prior to its consumption, an age-old food habit, not only provides a source of calcium to peoples not consuming milk or milk products, but also apparently provides or liberates a source of the vitamin, niacin.

SUMMARY

1. The growth depression of rats fed a high-corn diet, in the absence of niacin, was reduced by steeping the corn in

lime water, then neutralizing and drying it, a process similar to the one commonly used by generations of Mexican peasants in making "tortillas."

2. Corn reconstituted from the fractions obtained on wet-milling did not show this enhanced effect, being no different in its effects from the untreated material.

3. The full effect of the lime-water treatment was obtained by treatment of the non-starchy fractions — gluten, germ, and feed meal.

ACKNOWLEDGMENTS

We are grateful to Messrs. Wm. Wotherspoon and Co. of Paisley, Scotland, for providing us with corn products from their wet-milling plant. We are also indebted to Dr. Kodicek for his advice in the course of this work, to Dr. F. Stare for valuable criticism, and to Miss M. Robertson of the Statistical Department of this Institute for her analysis of the results of the experiments. One of us (J. L.) was in receipt of a World Health Organization Fellowship.

LITERATURE CITED

CHANDURI, D. K., AND E. KODICEK 1950 The biological activity for the rat of a bound form of nicotinic acid present in bran. Biochem. J., *47:* xxiv.

CRAVIOTO, B. R., R. ANDERSON, E. E. LOCKHART, F. DE P. MIRANDA AND R. S. HARRIS 1945 Nutritive value of Mexican tortilla. Science, *102:* 91.

HENDERSON, L. M., T. DEODHAR, W. A. KREHL AND C. A. ELVEHJEM 1947 Factors affecting the growth of rats receiving niacin-tryptophan-deficient diets. J. Biol. Chem., *170:* 261.

JACOBS, M. B. 1944 The Chemistry and Technology of Food and Food Products. Interscience Publishers, Inc., New York, N. Y., *II:* 464.

KODICEK, E. 1951 The biological activity for the rat of the bound form of nicotinic acid present in maize. Biochem. J., *48:* vii.

KREHL, W. A. 1949 Niacin in amino acid metabolism. Vitamins and Hormones, *7:* 111.

THOMSON, W. 1936 Stock diet for rats. J. Hygiene, *36:* 24.

THE EFFECT OF KIND AND LEVEL OF PROTEIN IN THE DIET ON THE PRODUCTION OF SOFT AND SKELETAL TISSUES[1]

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FOUR FIGURES

(Received for publication February 3, 1951)

The relationship between the level of protein intake and the utilization of calcium in the body has been the subject of animal experimentation over a period of years. In 1936 Conner and Sherman reported that with diets of liberal calcium content, increasing the protein intake increased the rate of growth of the animals and also the rate of calcification. This was in contrast to work reported by Fairbanks and Mitchell ('36) earlier in the same year, in which they stated that the calcium content of animals was inversely proportional to the rate of growth. They believed this inverse ratio to be the result of two tendencies — the tendency for rapid growth to be associated with slow calcification of the bones, and the tendency for rapid growth to be associated with high ratios of soft tissue to skeletal tissue. Fairbanks and Mitchell reported a direct ratio to exist between the calcium content of the body and the calcium content of the diet in cases where the dietary calcium was inadequate for maximum storage. In subsequent work Conner, Kao and Sherman ('41) also stated that there appeared to be no evidence that the increased gain in body weight which resulted from higher protein intakes had any accelerating effect on skeletal development as reflected in the percentage

[1] This study was supported in part by a grant from the Evaporated Milk Association on behalf of the American Dairy Association.

of body calcium. On increasing levels of protein, of from 14 to 25%, growing animals showed increasing body weight but decreasing percentages of total body calcium. The differences were wiped out, however, by the time the animals reached maturity. In recent work on this subject from Sherman's laboratory (Sherman et al., '47a, b) the idea has been advanced that on low-level calcium diets an increase in protein may cause a retardation of the body's normal developmental gain in calcium content. These workers believe that this may be regarded as an effect of protein-calcium imbalance, or of an undue acceleration of growth, or of both. The effect is not observed in animals on high calcium diets.

The study to be reported here was planned with the idea of comparing not only quantities but qualities of protein in their possible effect on the utilization of calcium. The proteins of milk and of wheat were used, both as they occur in natural foods and in the forms of casein and gluten. The effect of the purified proteins was studied at both high and low levels of calcium intake.

EXPERIMENTAL

Diets

The study was divided into three experimental periods. Two sources of protein were fed at levels of approximately 9 and 27%, making a total of 4 experimental diets in each period.

Period I. The protein was supplied by dry whole milk or by whole wheat, supplemented, where necessary to secure the required protein level, by casein or gluten. In the case of the 27% wheat protein diet, it was necessary to use a large proportion of gluten, making the diet perhaps more comparable to the synthetic diets than to the natural diets. The calcium level in all diets was adjusted to approximately the amount occurring naturally in the diet containing 27% milk protein.

Period II. The protein was supplied by casein or by gluten. The calcium was adjusted to approximately the same level as in period I.

Period III. The protein was supplied by casein or by gluten. The calcium was reduced to approximately one-fourth of the level fed in periods I and II.

An effort was made to have all the diets similar, the 27% milk protein diet in period I being taken as a guide. Thus, lactose and butterfat were included in all the diets, and specially prepared salt mixtures, containing the same amounts of various mineral elements as were calculated to be present in the milk contained in the standard diet, were used in the synthetic diets in periods II and III. The calcium : phosphorus ratio was approximately 1:1 in all the diets, and all of them provided approximately 4.5 cal. per gram of food. A mixture of synthetic vitamins was added to all the diets.[2] The vitamin supplement was larger and more varied in periods II and III, however, where only negligible quantities of vitamins were supplied by the ingredients of the diets. A 50% increase in the vitamin supplement was also made in the 27% wheat and gluten diet in period I, where the proportion of vitamin-containing foods was low.

Calculations with respect to the composition of the diets were based on the results of analyses of each of the principal foods in the diets for nitrogen, calcium, and phosphorus. Following thorough blending of the ingredients in a Hobart mixer, samples of the finished diets were analyzed for these same constituents. Sufficient quantities of food for an entire experimental period were mixed at one time, and were held at freezing temperatures. Portions sufficient for two or three days' feeding were removed and held at ordinary refrigerator temperatures until used.

Information on the composition of the diets is given in table 1.

Animals and feeding methods

Sixty albino rats of the Sprague-Dawley strain were used in each experimental period. At 28 days of age 5 groups of animals, comparable from the standpoint of weight, sex, and litter, were selected. One group of 12 was sacrificed, to serve as controls. Each of the remaining groups received one of the 4 experimental diets. Five animals in each group were

[2] We are indebted to Merck and Company for a supply of vitamins.

TABLE 1

Composition of diets[1]

PERIOD	I				II				III			
General character of diets	Natural foods, high Ca				Purified proteins, high Ca				Purified proteins, low Ca			
Diet code	m-Ca	M-Ca	w-Ca	W-Ca	c-Ca	C-Ca	g-Ca	G-Ca	c-ca	C-ca	g-ca	G-ca
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Whole dry milk	37.3	74.7	...	...	...	...	...	...	...	...	...	...
Casein	...	10.4	...	...	10.3	31.0	...	...	10.3	31.0	...	...
Whole wheat flour	...	...	45.8	17.6	...	...	...	...	..	...	...	...
Gluten	...	...	3.1	28.8	...	...	11.3	34.0	...	...	11.3	34.0
Lactose	16.5	...	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0
Butter fat	9.5	...	18.0	18.6	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0
Cornstarch	30.5	10.2	...	1.0	36.5	15.8	35.6	12.9	38.5	17.9	37.5	14.9
Cod liver oil	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Choline	0.10	0.10	0.10	0.10	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
$CaHPO_4 \cdot 2H_2O$	1.5	...	2.8	2.9	2.7	2.7	2.7	2.6	0.7	0.63	0.64	0.53
$NaHPO_4 \cdot H_2O$	0.7	0.6	..	..	0.6	0.5	0.5	0.3	0.2	0.1	0.1	...
Fe citrate	0.03	0.03	0.02	0.02	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
NaCl	2.0	2.0	2.0	2.0	0.8	0.9	0.9	1.1	1.0	1.0	1.1	1.2
KCl	..	..	..	..	0.4	0.4	0.4	0.6	0.6	0.7	0.7	0.7
K_2SO_4	..	..	..	..	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
$MgSO_4 \cdot 3H_2O$	..	..	..	..	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Protein (%)	9.0	26.4	9.3	25.9	9.2	27.6	9.0	27.6	9.8	26.7	9.5	27.2
Calcium (%)	0.78	0.69	0.87	0.87	0.86	0.74	0.88	0.82	0.19	0.26	0.17	0.17

[1] The diets were supplemented with the following amounts of synthetic vitamins per 100 gm:

Vitamin	*Period I*	*Periods II and III*	*Vitamin*	*Period I*	*Periods II and III*
	mg	*mg*		*mg*	*mg*
Thiamine	0.5	0.8	Calcium pantothenate	2.9	5.0
Riboflavin	1.0	1.6	Folic acid		0.32
Pyridoxine	0.5	0.8	Biotin		0.02
p-Aminobenzoic acid	4.8	8.0	Alpha-tocopherol		10.0
Nicotinic acid	3.0	4.8	Vitamin K		0.8
Inositol	2.4	20.0			

allowed to eat ad libitum; the other 7 were restricted to the food intake of the poorest feeder in all of the groups in that period.

The animals were housed in individual cages with one-half-inch wire mesh bottoms. Distilled water was supplied ad libitum. Any spilled food was collected each day, and records of food intake were kept. Animals were weighed and food intakes adjusted at three- or 4-day intervals.

The animals were maintained on the experimental diet for a period of 28 or 29 days. At the end of this time they were allowed to fast with free access to water for 24 hours. They were then killed by chloroform, and the internal organs were examined for gross abnormalities. The femurs were dissected free, sealed in moisture-proof cellophane, and kept frozen until analyzed.

Analyses

The two femurs of each animal were handled as a unit. They were measured with calipers, dried 48 hours at 60°C., extracted with alcohol and ether according to the method of Outhouse and Mendel ('33) and ashed in a muffle furnace. All calcium analyses on both femurs and food samples were carried out according to the McCrudden method as modified by Halliday and Noble ('43).

All analyses of food samples were based on the air-dry weight. Nitrogen determinations were carried out by the Kjeldahl method. All protein figures reported here are on the basis of $N \times 6.25$. Phosphorus determinations on food samples were made according to the method of Subbarow and Fiske (Halliday and Noble, '43), using a Coleman photoelectric colorimeter.

RESULTS

Food consumption and growth

Data on the average food consumption of the different experimental groups are presented in the form of bar graphs in figures 1 and 2. With the handling methods adopted, it

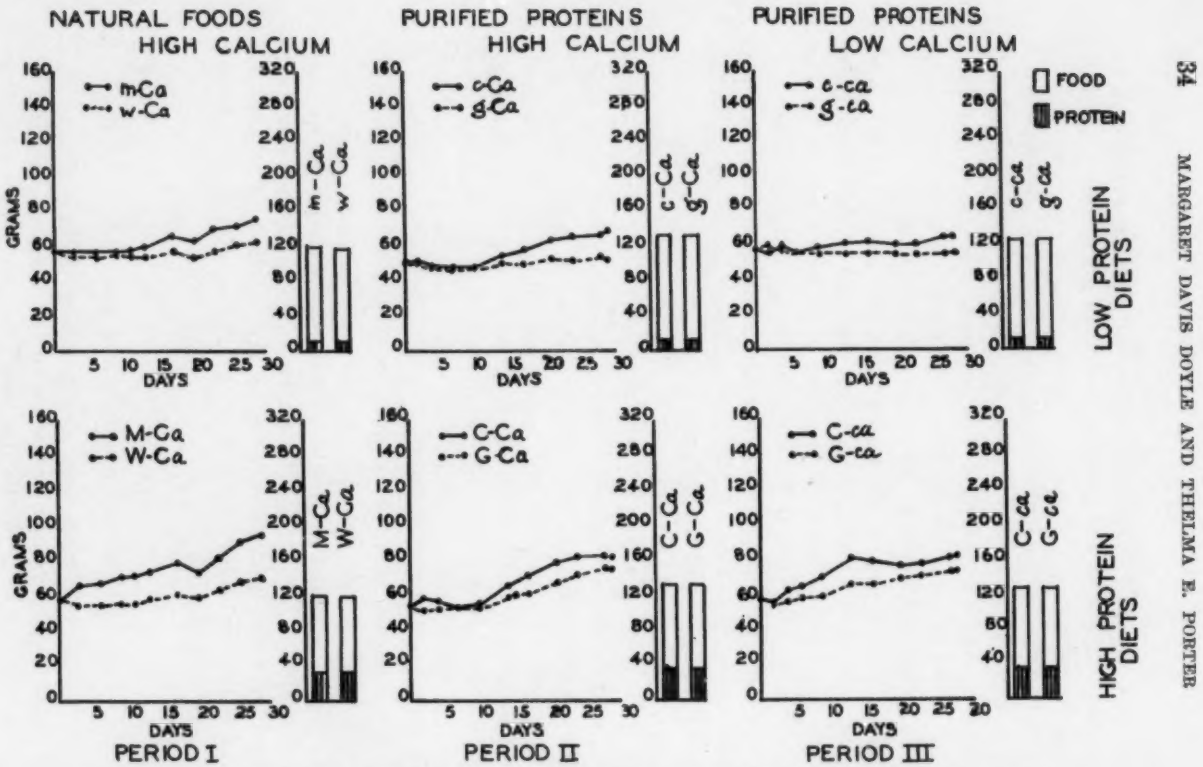

Fig. 1 Average weights of animals on restricted feeding, and average intakes of total food and of protein during experimental periods.

mental periods.

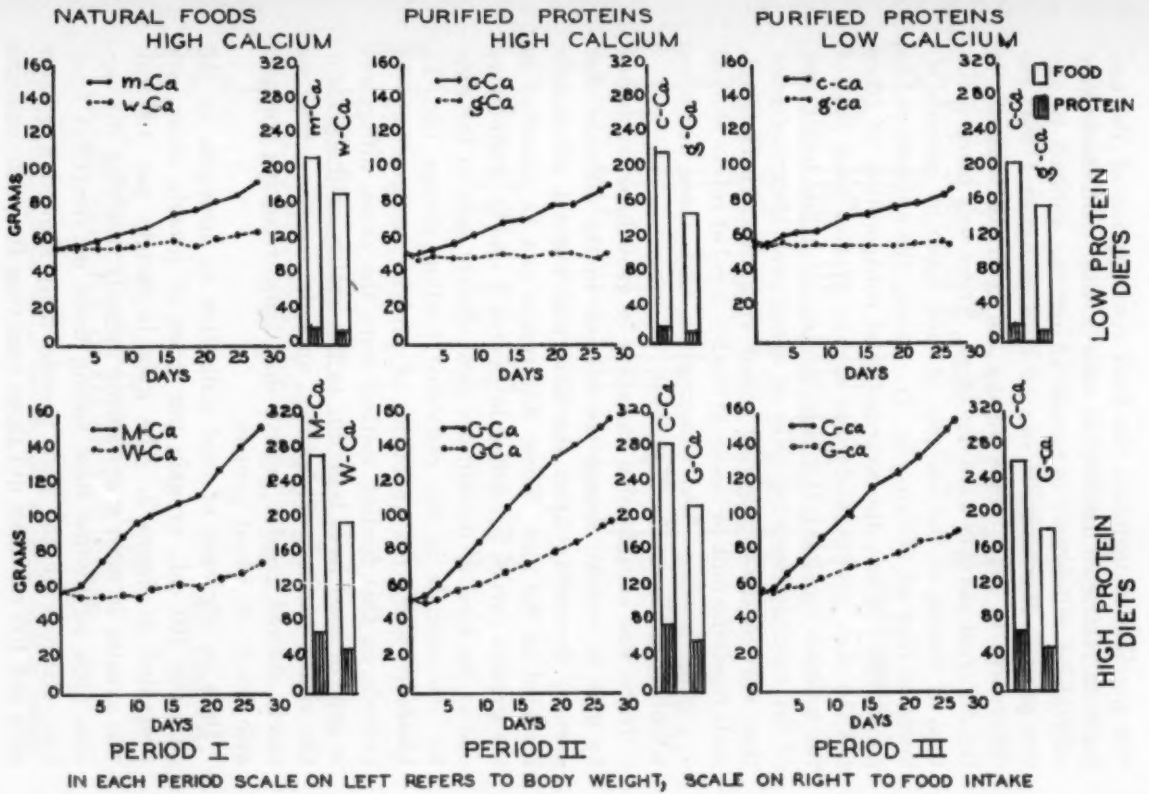

Fig. 2 Average weights of animals fed ad libitum, and average intakes of total food and of protein during experimental periods.

was possible to maintain the food consumption of the animals on restricted feeding in such a way that there were only slight differences in intake among the animals in any one period. Moreover, it can be seen in figure 1 that the differences in food intake among the restricted animals in the different periods were not large. There was more variation, of course, in the amounts of food taken by animals allowed to feed ad libitum (fig. 2). Among the animals on the ad libitum regime, the average food consumption of those fed the high protein diets was always greater than that of the animals receiving the same protein at a low level; that of the animals receiving milk or casein was always greater than that of those receiving wheat or gluten. In fact, animals receiving milk or casein at the 9% level of intake showed a greater average food consumption than those receiving wheat or gluten at the 27% level.

There was considerable variation in growth, as measured by gain in weight, among the animals in the different diet groups, depending upon the kind and amount of protein included in the diet. These differences can be observed in the growth curves presented in figures 1 and 2. Differences due to the kind of protein are most clearly seen in the data for the animals in the restricted feeding groups (fig. 1), however. Since the quantities of protein consumed by the animals on this feeding method were the same, differences in growth must have been due to the quality of the protein. On either method of feeding, animals receiving milk or casein showed greater growth than those receiving comparable levels of wheat protein.

Data on efficiency of food utilization as measured by the gain per 100 cal., or gain per gram of protein eaten, are presented in figure 3. The gain in weight per 100 cal. was greater in most cases among animals receiving the various diets ad libitum than among those on restricted feeding. Animals on the high protein diets showed a greater gain per 100 cal. than did those receiving the same proteins

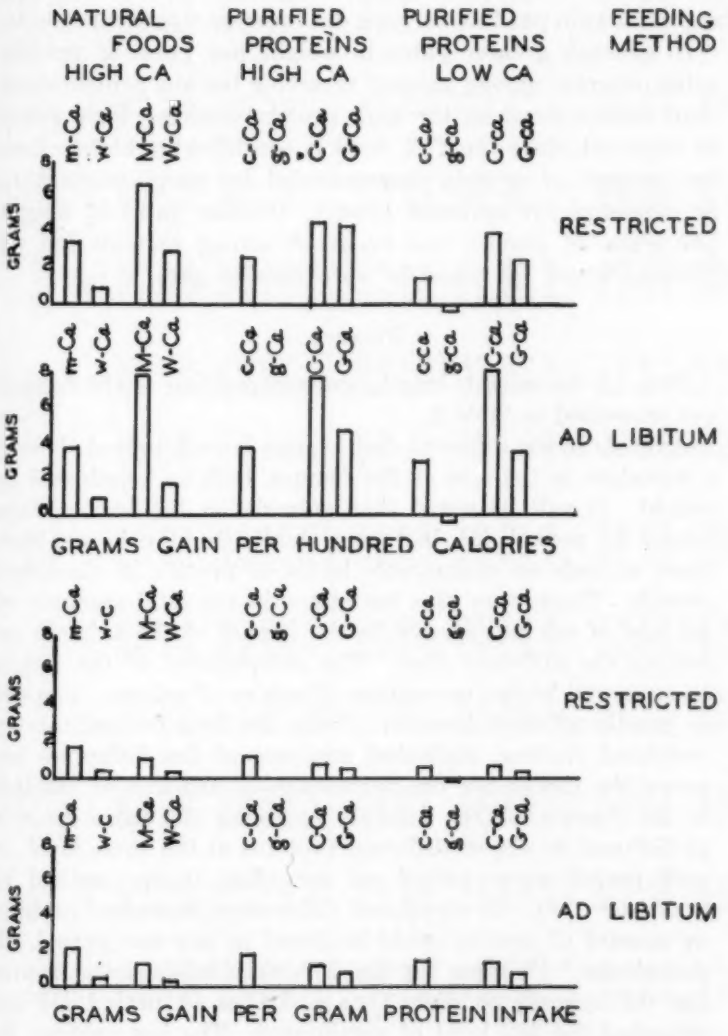

Fig. 3 Grams gain per 100 cal. and per gram of protein eaten by animals on restricted and ad libitum feeding regimes.

at the low level, and animals on "good" proteins showed a greater gain per 100 cal. than did those on "poor" proteins.

In general, greater gains in weight per gram of protein eaten occurred among animals receiving the low protein diets than among those on the high protein intakes. This would be expected, since the 27% level is considerably higher than the amount of protein demonstrated by many workers to be necessary for optimum growth. Greater gains in weight per gram of protein also occurred among animals fed ad libitum, where the animals were free to gain.

Femurs

Data on the weight, length, and composition of the femurs are presented in table 2.

Animals in the different diet groups in each period showed a variation in the size of the femurs, both in length and in weight. It will be noted that animals on the low calcium intake in period III had consistently lighter bones than those animals on comparable levels of protein in the other periods. There were also variations in the total amounts of ash and of calcium present in the femurs of the animals receiving the different diets. The composition of the bones, as measured by the percentage of ash or of calcium, was not so greatly affected, however. Using the data for animals on restricted feeding, statistical analyses of the difference between the means for the percentage of ash and of calcium in the femurs of the animals receiving the same protein at different levels, or different proteins at the same level, in each period were carried out according to the method of Snedecor ('46). No significant differences dependent on kind or amount of protein could be found in any one period, although the "t" value for the difference between the figures for the animals on diets C-ca and G-ca in period III approached the 5% level of significance. The low calcium intake in period III did result in an altered bone composition, however. The percentage both of ash and of calcium in the

TABLE 2

Average femur length, weight and composition, and deposition of calcium per gram of calcium intake, by period and by feeding method

TABLE 2

Average femur length, weight and composition, and deposition of calcium per gram of calcium intake, by period and by feeding method

PERIOD AND FEEDING METHOD	DIET CODE	FEMUR LENGTH	WEIGHT OF EXTRACTED FEMURS	ASH IN EXTRACTED FEMURS		WEIGHT BONE MATRIX	CALCIUM IN EXTRACTED FEMURS		CALCIUM IN ASH	CALCIUM DEPOSITED IN PERIOD	TOTAL CALCIUM INTAKE	CALCIUM DEPOSITED PER GRAM Ca INTAKE
		cm	gm	gm	%	gm	mg	%	%	mg	gm	mg
I												
Re-	m-Ca	2.34	0.367	0.183	50.10	0.184	68.8	18.45	37.32	43.6	0.9	48.4
stricted	w-Ca	2.22	0.329	0.157	48.03	0.172	58.1	17.81	37.08	32.9	1.0	32.9
	M-Ca	2.49	0.428	0.222	50.91	0.206	80.3	18.91	36.85	55.1	0.8	68.9
	W-Ca	2.27	0.320	0.164	51.31	0.156	60.8	19.09	37.20	35.6	1.0	35.6
I												
Ad	m-Ca	2.40	0.405	0.213	52.65	0.192	78.9	19.51	37.08	53.7	1.7	31.6
libitum	w-Ca	2.22	0.295	0.153	52.84	0.142	58.2	19.77	37.09	33.0	1.5	22.0
	M-Ca	2.70	0.554	0.304	54.93	0.250	112.3	20.29	36.94	87.1	1.9	45.8
	W-Ca	2.29	0.330	0.168	50.61	0.162	62.0	18.73	37.03	36.8	1.7	21.6
Controls		1.93	0.180	0.070	39.13	0.110	25.2	14.04	35.86			
II												
Re-	c-Ca	2.29	0.336	0.172	51.15	0.164	64.5	19.18	37.51	42.4	1.1	38.5
stricted	g-Ca	2.15	0.278	0.141	51.10	0.137	53.4	19.26	37.70	31.3	1.2	26.1
	C-Ca	2.44	0.382	0.195	51.00	0.187	73.1	19.11	37.47	51.0	1.0	51.0
	G-Ca	2.36	0.356	0.176	49.66	0.180	66.3	18.66	37.58	44.2	1.1	40.2
II												
Ad	c-Ca	2.43	0.390	0.200	51.28	0.190	74.9	19.24	37.50	52.8	1.9	27.8
libitum	g-Ca	2.13	0.271	0.132	48.98	0.139	48.9	18.18	37.17	26.8	1.3	20.6
	C-Ca	2.78	0.575	0.299	51.96	0.276	110.7	19.21	36.98	88.6	2.1	42.2
	G-Ca	2.46	0.399	0.202	50.72	0.197	75.1	18.79	37.04	53.0	1.8	29.4
Controls		1.82	0.162	0.062	38.32	0.100	22.1	13.61	35.51			
III												
Re-	c-ca	2.30	0.250	0.112	44.98	0.138	41.8	16.79	37.34	16.6	0.2	83.0
stricted	g-ca	2.16	0.228	0.102	45.00	0.126	37.7	16.56	36.83	12.5	0.2	62.5
	C-ca	2.40	0.307	0.141	45.86	0.166	52.1	17.00	37.08	26.9	0.3	89.7
	G-ca	2.34	0.256	0.113	44.00	0.143	41.4	16.16	36.74	16.2	0.2	81.0
III												
Ad	c-ca	2.38	0.283	0.137	48.36	0.146	50.7	17.90	37.03	25.5	0.4	63.8
libitum	g-ca	2.21	0.222	0.102	45.83	0.120	38.5	17.31	37.78	13.3	0.3	44.3
	C-ca	2.69	0.429	0.207	48.26	0.222	75.4	17.58	36.42	50.2	0.7	71.7
	G-ca	2.40	0.286	0.131	45.72	0.155	47.4	16.62	36.34	22.2	0.3	74.0
Controls		1.94	0.175	0.070	39.95	0.105	25.2	14.42	36.09			

femurs of the animals in this period was significantly lower than that of the animals in either period I or II.

It is evident from the data that certain changes take place in the bones of maturing animals, regardless of whether growth, as measured by gain in weight, occurs. The femurs of the control animals killed at the age of 28 days contained less calcium and ash, both in terms of total amounts and of percentages, than did those of the animals in any of the experimental groups, even those receiving diets g-Ca and g-ca, where no gain in weight occurred.

By subtracting the average amount of calcium present in the femurs of the control animals, killed at 28 days of age, from the average amount present in the femurs of the corresponding experimental animals, an estimate of the amount of calcium deposited during the experimental period is obtained. Dividing this figure by the total amount of calcium eaten during the period gives the amount of calcium deposited in the femurs per gram of calcium eaten. These data are also presented in table 2. Here again, the animals on the low calcium diet differed from those in the other periods. The total amounts of calcium deposited were smaller, but, because of the smaller intake, the amount deposited per gram of calcium eaten was larger than among the animals in the other periods, indicating more efficient utilization of calcium at lower levels of intake.

Animals on the high protein diets consistently deposited more calcium per gram of calcium eaten than did those on low protein diets. On comparable calcium intakes, animals receiving milk or casein usually deposited more calcium per gram of intake than did those receiving wheat or gluten. The exception to this finding was among animals receiving the 27% casein diet ad libitum in period III. These animals failed to deposit as much calcium per gram of intake as did the animals on the 27% gluten diet, even though the total amount of calcium deposited was considerably larger. This is explained by the fact that these animals ate enough food so

that their calcium intake, although low, was proportionately much higher than that of the animals on the 27% gluten diet.

DISCUSSION

It is obvious from the data presented that the amount of protein in the diets affected the over-all growth of the animals, and that the calcium content of the diets affected the composition, as well as the size, of the femurs. It is, however, difficult to determine whether the kind or amount of protein had any effect on the utilization of calcium — except indirectly, through growth.

In considering the problem of relative growth, Huxley ('32) pointed out that the ratio between the intensity of growth of an organ and that of the body remains constant over long periods of the animal's life. If the ratio is constant, a straight line will be formed when body weight is plotted against organ weight on a double logarithmic scale. Needham ('43) has shown the applicability of this principle to entities of a chemical nature as well as to bodily parts. Zucker and Zucker ('46a, b) have used this method of plotting body weight against the weight of bone ash or of bone matrix to illustrate certain nutritional effects on bone. They point out that there are three types of possible reaction of bone growth to nutritional deficiencies: (1) the growth of a bone constituent may be inhibited to the same extent as the growth of the body; (2) the bone constituent may be more markedly retarded than body weight; and (3) the bone constituent may be less affected than the body weight.

In the present study, if there were any effect of the protein, other than on total growth, it would be most likely to be detected in the animals on restricted feeding, where the actual amounts of protein eaten were similar. Data on the weight of the femurs and on the weight of the calcium, bone ash, and bone matrix (extracted femur weight minus the weight of the ash) in the femurs were plotted against the final body weight of the animals in the restricted feeding

groups on both high and low levels of calcium intake. Regression lines were calculated, and are presented in figure 4, together with the points for the average weight data for each of the above criteria. The y-intercepts of the lines for

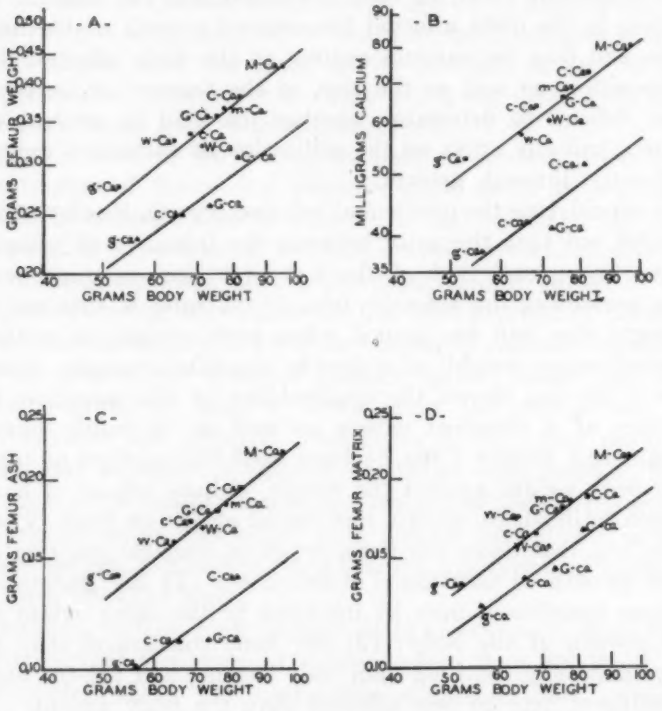

Fig. 4 Average final body weights of each group of animals, on restricted feeding, plotted on a logarithmic scale against the average weights of the femurs and of the femur ash, calcium, and matrix.

the animals on the low calcium diets were significantly different for each factor studied from those of the animals on the higher intakes of calcium. The slopes of the lines did not vary significantly, however.

When the data for the animals on the higher calcium intakes are considered, it will be noted that the only factor for which any of the animals showed marked deviation from regression was that of the weight of the bone matrix (fig. 4, chart D). The average point for the animals receiving diet W-Ca in period I falls below the regression line, that for animals receiving diet w-Ca in the same period falls above the regression line. In chart A (fig. 4), the average point for the femur weight of the animals receiving diet W-Ca is also somewhat below the line, that for animals receiving diet w-Ca is somewhat above the line. It would appear that any deviation from the "normal" or "expected" (as judged by the regression line for the group) in the size of the bones of these animals was due to differences in the organic, rather than the inorganic, constituents of the bones. In general, the close adherence of the points to the regression lines for all the factors suggests that, for animals receiving calcium at this level of intake, the kind or amount of protein has little, if any, effect on the utilization of calcium in the skeletal tissues.

When the data for the animals on the low calcium intakes are considered, it is observed that the point for the average weight of the femurs of the animals receiving diet G-ca falls below the line, that for animals receiving diet C-ca is above the line. These deviations in the weight of the femurs appear to be due to differences in the inorganic constituents of the bone, since the deviations from regression are more marked for bone ash and for calcium than for bone matrix. There appears to be some evidence, therefore, that on low levels of calcium intake the kind of protein may affect the utilization of calcium, particularly on high levels of protein intake, which produce greater over-all growth.

In addition to the evidence for a direct effect of protein on calcium utilization presented in the preceding paragraph, further evidence is found in the first section of table 3, in which the average figures for the animals on the high casein and high gluten diets in period III are compared. Al-

though the animals on diet C-ca had a final average weight which was only 12% greater than that of the animals on diet G-ca, their femurs were 20% heavier and contained 25% more ash and 26% more calcium than did those of the animals receiving gluten. This appears to indicate that animals receiving the high-casein, low-calcium diet had femurs which were somewhat larger and better calcified than might have been expected under the conditions of their growth.

TABLE 3

Average body weight, weight of femurs, and weight of calcium and ash in the femurs, with the percentage difference among these factors for certain groups of animals

PERIOD	DIET CODE	FINAL BODY WEIGHT	% DIFFERENCE	WEIGHT OF FEMURS	% DIFFERENCE	WEIGHT FEMUR ASH	% DIFFERENCE	CALCIUM IN FEMURS	% DIFFERENCE
		gm		*gm*		*gm*		*mg*	
III	C-ca	81.4	+ 12	0.307	+ 20	0.141	+ 25	52.1	+ 26
	G-ca	72.7	..	0.256	..	0.113	..	41.4	..
I	w-Ca	63.0	..	0.329	+ 3	0.157	..	58.1	..
	W-Ca	71.1	+ 13	0.320	..	0.164	+ 4	60.8	+ 5
III	g-ca	56.3	..	0.228	..	0.102	..	37.7	..
	G-ca	72.7	+ 29	0.256	+ 12	0.113	+ 11	41.4	+ 10
III	C-ca	81.4	+ 23	0.307	+ 23	0.141	+ 26	52.1	+ 25
	c-ca	66.4	..	0.250	..	0.112	..	41.8	..

The influence of the kind or amount of protein on bone formation may also be considered from the standpoint of possible indirect effects. Protein might be considered to have an indirect effect in bone formation if it brought about a disproportionate growth of soft tissue in relation to skeletal tissue. In the second section of table 3 average figures for the animals in periods I and III, on the diets in which a high level of protein was provided by wheat or gluten or both, are compared with the figures for animals on the lower level of the same proteins. Animals on diet W-Ca had a final average weight which was 13% greater than that of the animals receiving diet w-Ca. There was essentially no difference between the two groups in the size of the femurs, however. In this case, any advantage of the higher level

of protein seems to have been in the formation of soft tissues. In period III, animals on the high gluten diet had somewhat larger bones than did those on the low gluten diet. The difference in the bone size and composition was only in the range of 10 to 12%, however, while the difference in the average body weight was 29%.

It is recognized that whenever there is a limiting factor for bone growth, such as a low level of calcium intake, there may also be a disproportionate relationship between the skeletal and the total body weight. This is also illustrated in these data. All of the animals on the low calcium diets in period III may be considered to have had an overgrowth of soft tissue in relation to skeletal tissue if they are compared with the animals in periods I and II. A comparison of the animals on the high and low casein diets in period III (section 3, table 3) shows differences in body weight, and in femur weight and composition, which are approximately equal. This would indicate that the tendency for a disproportionate increase in body weight in relationship to bone weight did not occur with an increase in casein intake in the way that it did with an increase in gluten intake; and can be considered as providing further evidence of a specific beneficial effect of casein on bone growth.

SUMMARY AND CONCLUSIONS

1. The effect of variations in the kind and amount of protein intake upon the calcium utilization of growing albino rats was studied. Whole milk, whole wheat, casein and gluten, fed singly or in certain combinations, were the sources of protein. Protein was supplied at levels of approximately 9 and 27%. In one of the three experimental periods the amount of calcium present in the diets was approximately one-fourth the amount given in the other two periods. Both ad libitum and restricted feeding methods were employed.

2. The final weights of the animals varied considerably, depending upon the amount and kind of protein present in

the diet. Animals on the low calcium diets had lighter femurs, containing a smaller percentage of ash and of calcium than did the others.

3. A study of the data from animals on restricted feeding indicates that on low levels of calcium intake, casein tends to favor bone formation to a greater extent than does gluten. This may be considered a direct effect of protein on bone formation. Protein may also exert an indirect effect on bone formation by promotion of a growth of soft tissue which is out of proportion to the amount of skeletal tissue. Such an indirect effect of protein was observed in this study when relatively high levels of wheat or gluten, or both, were fed.

LITERATURE CITED

CONNER, R. T., H. KAO AND H. C. SHERMAN 1941 Further studies on the relationship of the plane of protein intake to the rate of normal calcification during growth. J. Biol. Chem., *139:* 835.

CONNER, R. T., AND H. C. SHERMAN 1936 Some aspects of protein intakes in relation to growth and rate of calcification. Ibid., *115:* 695.

FAIRBANKS, B. W., AND H. H. MITCHELL 1936 The relation between calcium retention and the store of calcium in the body, with particular reference to the determination of calcium requirements. J. Nutrition, *11:* 551.

HALLIDAY, E. G., AND I. T. NOBLE 1943 Food Chemistry and Cookery. The University of Chicago Press, Chicago, Ill.

HUXLEY, J. S. 1932 Problems of Relative Growth. Methuen and Company, Ltd., London.

NEEDHAM, J. 1943 Chemical heterogony and the ground plan of animal growth. Biol. Rev. Biol. Proc., *9:* 79.

OUTHOUSE, J., AND L. B. MENDEL 1933 The rate of growth. I. Its influence on the skeletal development of the albino rat. J. Exp. Zool., *64:* 257.

SHERMAN, H. C., AND M. S. RAGAN 1947a Further studies of the influence of nutrition upon the chemical composition of the body. Proc. Natl. Acad. Sci., *33:* 266.

SHERMAN, H. C., M. S. RAGAN AND M. I. BAL 1947b Effect of increasing food protein upon the calcium content of the body. Ibid., *33:* 356.

SNEDECOR, G. W. 1946 Statistical Methods. Iowa State College Press, Ames.

ZUCKER, L. M., AND T. F. ZUCKER 1946a Specific and non-specific nutritional effects as illustrated in bone. Am. J. Physiol., *146:* 593.

ZUCKER, T. F., AND L. M. ZUCKER 1946b Bone growth in the rat as related to age and body weight. Ibid., *146:* 585.

AN INTERRELATION OF PTEROYLGLUTAMIC ACID, VITAMIN B_{12} AND THE MONKEY ANTI-ANEMIA FACTOR [1]

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TWO FIGURES

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Experiments in this laboratory have demonstrated that monkeys deficient in riboflavin, pyridoxine, pantothenic acid or pteroylglutamic acid (PGA) do not respond completely to therapy with the missing vitamin (Cooperman et al., '45b; McCall et al., '46; Cooperman et al., '46a). An initial response in weight and blood regeneration occurs, but this is followed by a condition where further improvement results only upon the administration of certain liver products or milk (Cooperman et al., '45a, '46b). The efficacy of such crude products has been attributed to a substance tentatively named the monkey anti-anemia factor (MAAF), and it has been suggested that a deficiency of this factor is precipitated concomitantly with a deficiency of any one of the 4 aforementioned vitamins. This condition has also been produced by the inclusion of 40% of corn grits in the diet (Ruegemer et al., '48). The MAAF deficiency syndrome is characterized by anemia, reversal of the neutrophile-lymphocyte (N/L) ratio, and lack of growth.

Schaefer et al. ('48a, b) showed that the mink and fox require, in addition to all the known vitamins, an unidentified factor present in a 60% methanol extract of liver (MEL).

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Later Tove et al. ('49) reported that the MEL had a sparing effect on the dietary PGA requirement of the mink. This effect could not be achieved by the daily injection of 1 μg of vitamin B_{12}, but more recently partial responses were obtained by the injection of 10 μg per day or by feeding 20 μg (Tove et al., '50). In the present paper we report the effects of MEL, vitamin B_{12} and high levels of PGA in the treatment of PGA-defiicent monkeys.

EXPERIMENTAL AND RESULTS

The housing, care and handling of the monkeys have been described previously (Waisman et al., '43). The basal ration was composed of sucrose 73, casein [2] 18, salts IV 4 and corn oil [3] 5%. Daily vitamin supplements included thiamine hydrochloride 0.5 mg, riboflavin 0.5 mg, niacin 5 mg, pyridoxine hydrochloride 1 mg, calcium pantothenate 3 mg, biotin 0.02 mg, choline chloride 25 mg, *i*-inositol 50 mg, sodium *p*-aminobenzoate 100 mg, ascorbic acid 25 mg, and PGA 0.1 mg. A PGA deficiency was created by omitting this vitamin from the supplements. MEL was administered by stomach tube as the monkeys refused to consume it voluntarily. Vitamin B_{12} was injected intraperitoneally.

As has been commonly noted in several laboratories, PGA-deficient monkeys are very susceptible to dysentery. When this condition developed, sulfasuxidine was given with the vitamin supplements. The animals were weighed weekly until a marked weight loss was noted, after which the weighings were increased to two or three weekly. Blood examinations were made at appropriate intervals throughout the experimental periods on samples taken from the saphenous vein of the leg. Hemoglobin (Hb) was determined in the Evelyn photoelectric colorimeter, and white blood cell (WBC) and red blood cell (RBC) counts were made in the usual manner. Differential WBC counts were made on preparations stained with Wright's stain. The criteria used in determining when to in-

[2] Vitamin-test, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Mazola fortified with oleum percomorphum (Mead Johnson and Co., Evansville, Ind.) to supply vitamins A and D.

itiate therapy were a marked weight loss, anemia, leukopenia, and reversed N/L ratio. Occasionally all of these standards could not be met when the condition of the monkey became so critical as to preclude continuation of the deficient diet. Growth and blood data are contained in table 1.

Control animals

Two monkeys (numbers 453 and 454) were kept on the basal ration with complete vitamin supplements for a period of 62 weeks. During this time a normal blood picture was maintained, and the growth rate was good. This also confirms the findings of Cooperman et al. ('46a) and of Day and Totter ('48) that 100 μg of PGA per day are adequate for the normal, young rhesus monkey.

PGA studies

Four monkeys (numbers 456, 458, 459 and 460) received the basal ration with all vitamin supplements except PGA. Dysentery occurred without exception as the deficiency progressed, and was counteracted in some instances by sulfasuxidine.

PGA therapy was initiated at a level of 500 μg per day, which effected a normal N/L ratio in from 9 to 14 days and alleviated the leukopenia. Monkeys 456, 458 and 459 exhibited increases in Hb concentration, but the RBC count rose to a normal value only in monkey 458. At the end of 7 to 8 weeks, these three animals had made weight gains ranging from 290 to 605 gm. It was necessary to feed monkey 460 by stomach tube during most of the three-week period in which it received 500 μg of PGA daily, and little improvement was obtained. Upon increasing the PGA to 1 mg per day the dysentery cleared up, a normal blood picture was established, and a weight gain of 725 gm in 6 weeks resulted. The blood pictures of animals 456, 458 and 459 were improved somewhat by increasing the PGA to 1 mg per day. The data for monkey 458 are presented graphically in figure 1. In an earlier experiment it was found that 1 mg of PGA per day was the minimum

TABLE 1

Data showing growth and hematological responses by PGA-deficient monkeys to PGA, MEL and vitamin B_{12}

MONKEY NO.	SUPPLEMENT	WEEKS	WEIGHT	Hb	RBC	WBC	N/L RATIO NORMAL
			gm	*gm %*	*million per mm³*	*thousand per mm³*	
			Controls				
453 ♂	100 μg PGA/day	0	3,870	12.6	4.5	17.0	Yes
		62	7,660	14.3	5.2	24.9	Yes
454 ♂	100 μg PGA/day	0	2,930	11.7	5.2	16.1	Yes
		62	6,645	13.1	5.1	15.5	Yes
			PGA therapy				
456 ♀	None	18	3,410	12.1	5.2	8.7	No
	500 μg PGA/day	8	3,790	12.6	4.3	20.6	Yes
	1 mg PGA/day	8	4,170	12.0	4.5	21.3	Yes
	100 μg PGA/day	15	4,525	11.6	4.3	22.1	Yes
458 ♀	None	18	3,265	11.8	2.7	10.7	No
	500 μg PGA/day	7	3,555	14.3	5.4	17.0	Yes
	1 mg PGA/day	8	3,795	14.4	6.0	16.5	Yes
	100 μg PGA/day	14	4,290	13.3	6.1	18.9	Yes
459 ♂	None	18	3,420	11.6	3.5	12.7	No
	500 μg PGA/day	8	4,025	12.8	3.9	21.1	Yes
	1 mg PGA/day	8	4,755	13.0	4.7	17.3	Yes
	100 μg PGA/day	15	5,890	11.6	4.1	14.7	Yes
460 ♀	None	4	2,945	12.4	4.3	8.4	No
	500 μg PGA/day	3	2,980	12.4	4.4	17.4	Yes
	1 mg PGA/day	6	3,705	14.3	4.9	19.6	Yes
			MEL therapy				
451 ♂	None	18	3,760	13.0	4.8	15.1	No
	1 ml MEL/2 days	4	3,775	13.2	4.6	11.8	Yes
	4 ml MEL/4 days	3	3,815	13.8	4.7	12.8	Yes
	4 ml MEL/2 days	8	4,325	13.6	3.0	18.8	No
	4 ml MEL/2 days + 50 μg PGA/day	7	4,985	15.5	7.0	29.5	Yes
	100 μg PGA/day	28	5,795	15.2	6.0	20.1	Yes
452 ♀	None	13	2,815	9.9	4.1	4.8	Yes
	1 ml MEL/2 days	4	3,325	12.7	4.7	9.8	Yes
	4 ml MEL/4 days	3	3,260	13.4	5.0	10.0	Yes
	4 ml MEL/2 days	8	3,550	13.4	4.0	17.0	No
	4 ml MEL/2 days + 50 μg PGA/day	7	4,000	14.2	5.0	14.4	Yes
	100 μg PGA/day	8	4,360	12.1	4.0	15.5	Yes
460 ♀	None	18	3,655	11.0	4.1	13.1	Yes
	1 ml MEL/2 days	4	3,825	12.3	3.5	12.0	No
	4 ml MEL/4 days	3	3,715	12.7	3.7	14.0	No
	4 ml MEL/2 days	8	4,025	13.2	4.0	11.9	Yes
	4 ml MEL/2 days + 50 μg PGA/day	7	4,285	13.9	4.9	17.6	Yes
	100 μg PGA/day	8	4,720	12.2	4.2	13.9	Yes

TABLE 1 *(continued)*

MONKEY NO.	SUPPLEMENT	WEEKS	WEIGHT	Hb	RBC	WBC	N/L RATIO NORMAL
			gm	*gm %*	*million per mm³*	*thousand per mm³*	
			MEL therapy				
496 ♂	None	18	2,500	14.3	5.5	8.6	No
	6 ml MEL/day	1	2,310	12.1	4.5	7.0	No
	4 ml MEL/2 days	12	3,595	13.5	4.3	16.2	Yes
	4 ml MEL/2 days + 25 μg PGA/day	4	3,810	12.1	4.4	11.0	Yes
	4 ml MEL/2 days + 50 μg PGA/day	13	4,180	13.0	5.8	13.0	Yes
	100 μg PGA/day	6	4,520	12.1	5.3	15.5	Yes
499 ♀	None	20	3,495	9.6	4.4	7.1	Yes
	4 ml MEL/2 days	13	4,240	12.8	4.2	19.5	Yes
	4 ml MEL/2 days + 25 μg PGA/day	4	4,490	12.2	4.0	13.6	Yes
	4 ml MEL/2 days + 50 μg PGA/day	13	5,325	12.6	5.0	16.6	Yes
	100 μg PGA/day	10	5,375	12.1	4.6	13.5	Yes
500 ♀	None	20	3,090	11.4	4.0	9.9	Yes
	4 ml MEL/2 days	13	3,625	12.1	3.3	18.8	Yes
	4 ml MEL/2 days + 25 μg PGA/day	4	3,835	11.3	3.4	16.4	Yes
	4 ml MEL/2 days + 50 μg PGA/day	13	4,470	11.7	4.9	24.6	Yes
			B_{12} therapy				
489 ♂	None	22	2,330	12.4	4.6	11.8	Yes
	1 μg B_{12}/day	4	2,130	13.9	5.3	18.7	No [1]
	1 μg B_{12} + 50 μg PGA/day	9	2,565	12.4	4.9	29.5	Yes
	100 μg PGA/day	5	2,745	10.4	4.3	27.9	No [1]
	1 mg PGA/day	11	3,070	10.5	4.7	27.3	Yes
	4 ml MEL/2 days	4	3,315	10.7	4.3	23.6	Yes
	4 ml MEL/2 days + 50 μg PGA/day	13	3,575	12.3	5.7	22.9	Yes
	100 μg PGA/day	6	3,830	12.4	5.6	20.5	Yes
494 ♀	None	14	2,000	10.9	2.2	6.0	No
	1 μg B_{12}/day	2	1,865	11.5	4.5	5.1	No [1]
	2 μg B_{12}/day	2	1,805	11.6	3.6	5.6	No
	1 μg B_{12} + 100 μg PGA/day	3	2,505	13.0	5.6	33.2	No
	100 μg PGA/day	18	3,400	9.8	3.2	23.4	No [1]
	1 mg PGA/day	11	3,585	11.1	4.6	11.7	Yes
	4 ml MEL/2 days	4	3,950	11.7	5.1	16.3	Yes
	4 ml MEL/2 days + 50 μg PGA/day	13	4,465	11.7	5.6	15.2	Yes
	100 μg PGA/day	10	4,735	12.1	5.3	14.6	Yes

[1] Ratio abnormal at end of period, but normal at some time during period.

amount tested that would cause complete remission of all deficiency signs in monkeys 458 and 459, which had been made PGA-deficient.

Studies with methanol extract of liver

Monkey 460, which had been used in the PGA studies, and 5 other monkeys (numbers 451, 452, 496, 499 and 500) were maintained on the PGA-deficient diet until signs of PGA deficiency were evident. Monkeys 451, 452 and 460 first re-

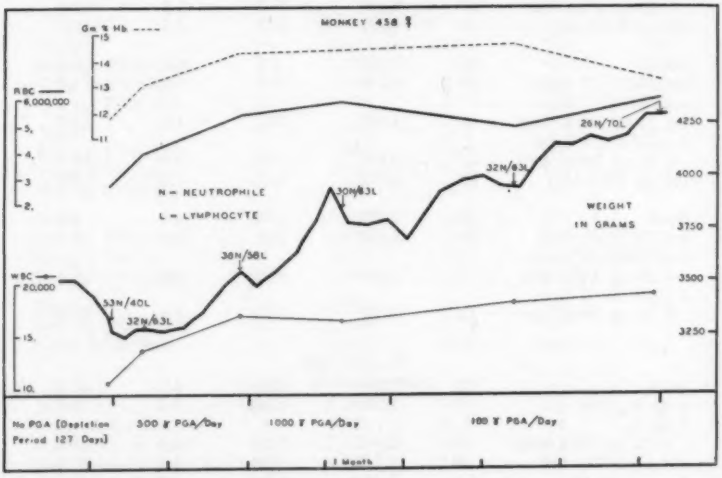

Fig. 1 (Monkey 458) The effect of high levels of PGA on the growth and blood picture of a PGA-deficient monkey.

ceived 1 ml of MEL (equivalent to 9 gm fresh liver) every two days. Although some improvement resulted, the responses were neither consistent nor optimum. Data for monkey 451 are presented graphically in figure 2. Rates of growth and blood pictures did not become normal until the MEL was increased to 4 ml every two days, and the animals received this therapy for about a month before any benefits were apparent. Shortly following the increase in weight there was deterioration of the blood pictures of monkeys 451 and 452,

marked by low RBC counts and reversed N/L ratios. In addition, dysentery developed in these two and in monkey 460, and the WBC count of the latter animal was low. With the addition of 50 μg of PGA per day, normal blood pictures and good weight gains were established, and the dysentery cleared up. After 7 weeks the MEL was discontinued, and the PGA was increased to 100 μg per day, which supported good growth and blood formation. At the end of 7 months monkey 451 was normal in every respect.

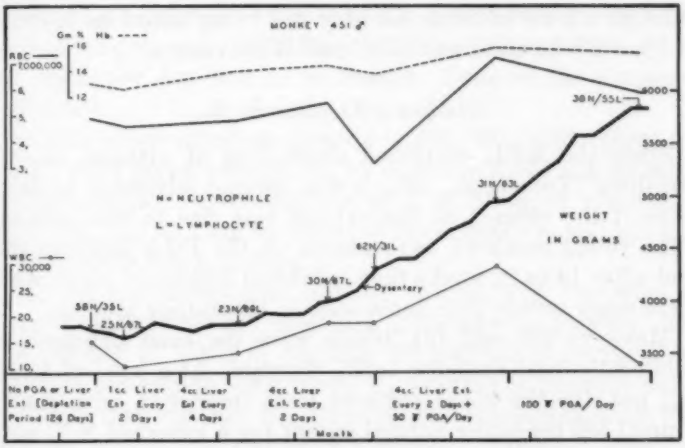

Fig. 2 (Monkey 451) The effect of methanol extract of liver on the growth and blood picture of a PGA-deficient monkey.

Monkey 496, after being on the PGA-deficient diet for 18 weeks, developed dysentery, became very weak and refused to eat. The only blood abnormalities were a reversed N/L ratio and leukopenia. When 6 ml of MEL were given per day, the dysentery cleared up and normal food consumption was resumed. The level of MEL was then reduced to 4 ml every two days for 12 weeks, which produced a weight gain of 1,285 gm and a more nearly normal blood picture. Further improvement in the blood picture resulted from the addition of 50 μg

of PGA per day. When the MEL was discontinued and the level of PGA increased to 100 μg per day, the weight gain and blood picture were satisfactory.

With monkeys 499 and 500, MEL was first given at a level of 4 ml every two days. This treatment produced good responses in weight gain, Hb value and WBC count. The RBC count increased for a time, then decreased to a value below that at the end of the depletion period. The addition of 25 μg of PGA per day not only was ineffective in increasing the RBC count, but also did not maintain the Hb concentration. PGA at a level of 50 μg per day did bring about an increase in Hb concentration and RBC and WBC counts.

Studies with vitamin B_{12}

Since the MEL contained about 2 μg of vitamin B_{12} per milliliter (Tove et al., '50), it was deemed advisable to determine if the efficacy of the extract was due to this vitamin. Nine young monkeys were placed on the PGA-deficient diet, and after 14 to 22 weeks they exhibited deficiency signs which were more severe than those seen in the earlier studies.

Monkeys 489 and 494, which were the least deficient, exhibited the best response to B_{12} therapy. At a level of 1 μg of B_{12} per day the blood pictures were improved, with that of animal 489 becoming normal except for a reversed N/L ratio. Increasing the level to 2 μg per day did not result in further improvement in monkey 494. The N/L ratio remained abnormal and anorexia was prevalent, necessitating frequent feedings by stomach tube. Both monkeys continued to lose weight during this therapy. The addition of 50 or 100 μg of PGA per day caused an increase in weight, but the blood pictures were not maintained by 100 μg of PGA alone per day. Some improvement was obtained by increasing the PGA to 1 mg per day, but the blood pictures were suboptimum. Four milliliters of MEL every two days produced no response in monkey 489 but did elicit a response in animal 494. A combination of 4 ml of MEL every two days and 50 μg of PGA per day improved

the blood picture of monkey 489, but the only effect on animal 494 was a slight increase in the RBC count. The weight gains and blood pictures were then maintained by 100 μg of PGA per day.

Four of 9 monkeys showed an initial response to vitamin B_{12}. The N/L ratios returned to normal in one week and the RBC counts, which ranged from 1.9 to 4.2 million per mm^3, increased to 3.1 to 5.4 million per cubic millimeter. However, the Hb concentration continued to fall and no weight gains were evident. These animals became progressively emaciated, weaker and refused to eat. The dysentery did not respond to sulfasuxidine, and the addition of 25 or 50 μg of PGA or 4 ml of MEL per day was of no benefit. None of the 4 monkeys survived.

Three other monkeys (numbers 495, 497, 498), when deficient, were given vitamin B_{12} by injection and PGA by mouth simultaneously. Combinations of 7 or 15 μg of vitamin B_{12} and 0.01, 0.2 or 1 mg of PGA were ineffective, and all three died in from two to 4 days.

Of the 9 monkeys used in these studies, only two were improved sufficiently by vitamin B_{12} therapy to survive. That the efficacy of MEL in treating PGA-deficient monkeys was not due entirely to its vitamin B_{12} content is brought out by the fact that vitamin B_{12} alone or in conjunction with PGA did not prevent the deaths of 7 animals. Four of these 7 exhibited some increase in RBC count and the N/L ratio became normal. Only one monkey showed an improvement in Hb concentration. All of the observed effects were less pronounced than those obtained with MEL, with the possible exception of those relating to animal 489, which was not greatly deficient at the time therapy was initiated. In no instance did vitamin B_{12} arrest the loss in weight.

DISCUSSION

The normal monkey apparently has no dietary requirement for the MAAF or vitamin B_{12}. The importance of these substances becomes apparent only under certain conditions of

nutritional stress. The fact that large amounts of PGA (10 times the minimum daily requirement) caused remission of the syndrome precipitated in monkeys by a deficiency of PGA and attributed to a lack of the MAAF suggests that the PGA was acting indirectly to replace a more fundamental or active principle. Consistently good responses in weight gain and blood picture were obtained with MEL, although this material contributed only about 4 μg of PGA per day (Tove et al., '49). Although the MEL could not indefinitely replace PGA in the diet of the monkey, the addition of only 50 μg of PGA per day maintained a normal blood picture. The results obtained with a relatively small amount of PGA when used in conjunction with MEL were duplicated only by a large amount of PGA alone. The MEL evidently exerted a sparing action on the dietary PGA requirement of the monkey.

The activity of the MEL cannot be attributed entirely to its content of vitamin B_{12}, since this vitamin was effective in eliciting responses only in monkeys that were not greatly deficient in PGA and was impotent with respect to the more deficient animals. Nevertheless, the fact that some response occurred makes necessary consideration of vitamin B_{12} as related in action to PGA and the factor or factors present in MEL. The effectiveness of the vitamin B_{12} may have been due to its ability to stimulate synthesis of PGA. Dietrich et al. ('49) found that B_{12} stimuated the growth of chicks fed a purified ration containing a low level of PGA and produced an increased amount of PGA in the liver. Addition of both vitamins C and B_{12} gave a greater growth response than either alone. The data of Register et al. ('50) indicate that the monkey is usually able to obtain sufficient B_{12} through intestinal synthesis even when deficient in PGA and vitamin C, although maximum synthesis is apparently favored by these substances. The fact that two animals responded to B_{12} in the present studies may indicate that an extra amount of the vitamin stimulated synthesis of PGA sufficiently to permit recovery. It is possible that other unknown factors in the MEL are also effective in stimulating synthesis of PGA. Tove

et al. ('49) found that mink receiving 1% of MEL per day showed the same increase in the PGA content of blood, urine and feces as did animals receiving 0.1 mg % of PGA per day. The MEL also produced an increase in the urine and fecal levels of PGA of the mink receiving 0.1 mg % of PGA.

Despite the fact that high levels of PGA satisfy any requirement for the MAAF under the conditions employed, it is unlikely that this substance is identical wth the citrovorum factor (CF) of Sauberlich and Baumann ('48), since Reticulogen, which is a good source of CF, is inactive in the monkey as a source of the MAAF (Ruegamer et al., '48) and does not replace methanol extract of liver in the nutrition of the mink (Tove et al., '49).

SUMMARY

1. A deficiency of PGA was created in young rhesus monkeys by means of a purified diet. Signs characteristic of a lack of the monkey anti-anemia factor accompanied those of the primary deficiency.

2. Remission of the monkey anti-anemia factor deficiency syndrome was obtained by the oral administration of PGA at a level 10 times the minimum daily requirement. Remission for a limited period was also obtained with a 60% methanol extract of liver supplying only 4 μg each of PGA and vitamin B_{12} per day. Eventually PGA had to be supplied to maintain normal growth rate and blood picture, but it was required in much smaller amounts than when the methanol extract of liver, which is a good source of the monkey anti-anemia factor, was not employed.

3. Vitamin B_{12} produced responses only in the less-deficient monkeys and probably acted in conjunction with vitamin C to stimulate the synthesis of PGA.

4. A relationship exists among PGA, vitamin C, vitamin B_{12} and the monkey anti-anemia factor, although the latter two substances are not ordinarily required in the diet of the normal monkey.

5. At high levels PGA appears to be capable of satisfying any requirement that may exist in a PGA-deficient monkey for vitamin B_{12} or the factor or factors present in methanol extract of liver (the monkey anti-anemia factor).

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LITERATURE CITED

COOPERMAN, J. M., C. A. ELVEHJEM, K. B. MCCALL AND W. R. RUEGAMER 1946a "Folic acid" active compounds in the nutrition of the monkey. Proc. Soc. Exp. Biol. Med., *61:* 92.

COOPERMAN, J. M., K. B. MCCALL AND C. A. ELVEHJEM 1945a Further studies on the monkey anti-anemia factor. Science, *102:* 645.

COOPERMAN, J. M., W. R. RUEGAMER AND C. A. ELVEHJEM 1946b Milk as a source of the monkey anti-anemia factor. Proc. Soc. Exp. Biol. Med., *62:* 101.

COOPERMAN, J. M., H. A. WAISMAN, K. B. MCCALL AND C. A. ELVEHJEM 1945b Studies on the requirements of the monkey for riboflavin and a new factor found in liver. J. Nutrition, *30:* 45.

DAY, P. L., AND J. R. TOTTER 1948 Pteroylglutamic acid balance studies on monkeys. Ibid., *36:* 803.

DIETRICH, L. S., C. A. NICHOL, W. J. MONSON AND C. A. ELVEHJEM 1949 Observations on the interrelation of vitamin B_{12}, folic acid and vitamin C in the chick. J. Biol. Chem., *181:* 915.

MCCALL, K. B., H. A. WAISMAN, C. A. ELVEHJEM AND E. S. JONES 1946 A study of pyridoxine and pantothenic acid deficiencies in the monkey (Macaca mulatta). J. Nutrition, *31:* 685.

REGISTER, U. D., U. J. LEWIS, D. V. TAPPAN AND C. A. ELVEHJEM 1950 Relationship of vitamin B_{12}, folic acid and vitamin C in the nutrition of the rhesus monkey. Unpublished data.

RUEGAMER, W. R., E. M. SPORN, U. D. REGISTER AND C. A. ELVEHJEM 1948 Distribution and fractionation of the monkey anti-anemia factor. J. Nutrition, *36:* 405.

SAUBERLICH, H. E., AND C. A. BAUMANN 1948 A factor required for the growth of *Leuconostoc citrovorum*. J. Biol. Chem., *176:* 165.

SCHAEFFER, A. E., S. B. TOVE, C. K. WHITEHAIR AND C. A. ELVEHJEM 1948a The requirement of unidentified factors for mink. J. Nutrition, *35:* 157.

SCHAEFER, A. E., C. K. WHITEHAIR AND C. A. ELVEHJEM 1948b Unidentified factors essential for growth and hemoglobin production in foxes. Ibid., *35:* 147.

TOVE, S. B., R. J. LALOR AND C. A. ELVEHJEM 1950 Properties of the methanol soluble factor required by the mink. Proc. Soc. Exp. Biol. Med., *75:* 71.

TOVE, S. B., A. E. SCHAEFER AND C. A. ELVEHJEM 1949 Folic acid studies in the mink and fox. J. Nutrition, *38:* 469.

WAISMAN, H. A., A. F. RASMUSSEN, JR., C. A. ELVEHJEM AND P. F. CLARK 1943 Studies on the nutritional requirements of the rhesus monkey. Ibid., *26:* 205.

THE NUTRITIVE EFFECT OF HEATING LACTALBUMIN IN THE PRESENCE OF VARIOUS REDUCING SUGARS[1]

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FOUR FIGURES

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In recent studies of the deleterious effect of heat processes upon the nutritional value of proteins, it has been proposed that part of the damage could be attributed to the interaction of the protein with reducing sugars present in the preparation. Hill and Patton ('47) demonstrated that the browning or Maillard reaction, which results from the interaction of glucose and the casein hydrolysate components of microbiological media when they are autoclaved, decreases the nutritional value of the media. Further studies showed that this decrease can be attributed to a partial inactivation of free amino acids due to the browning reaction (Patton and Hill, '48). The inactivation of protein-bound amino acids was shown to occur when casein was heated with glucose (Patton, Hill and Foreman, '48a). In another study of this reaction, Mader, Schroeder and Smith ('49) demonstrated that part of the nutritional damage resultant from heating commercial lactalbumin observed by Davis, Rizzo and Smith ('49) could be attributed to the interaction of this protein with the lactose present in the commercial preparation.

[1] The data in this paper are taken from the dissertation submitted by Robert A. Stewart for the degree of Master of Science, Wayne University, 1950. A preliminary report of the work was given before the Michigan Academy of Science at Ann Arbor, April 28, 1950.

The purpose of this investigation has been to confirm the effect of the interaction of lactose and lactalbumin, when heated, upon the nutritional value of this protein. To study further the effect of the Maillard reaction upon the value of this protein, the nutritional effect of heating it with other reducing sugars has been studied, since it has been shown that the extent of the reaction is proportional to the availability of free aldehyde groups (Ransey, Tracy and Ruehe, '33; Ross, '48). The investigation was conducted by a nitrogen balance study in dogs, to permit analysis of the effect of heat treatment upon the digestibility and biological value of these protein-sugar mixtures.

EXPERIMENTAL

The protein used in this study was commercial lactalbumin.[2] A large amount of reducing substances, primarily lactose, is found in this commercial preparation; these were removed by the water extraction process described by Mader, Schroeder and Smith ('49). The extracted protein was combined with glucose, fructose, lactose, maltose, and xylose to recreate the same mole per cent of reducing sugars as was present in the original commercial protein. Each of the protein-sugar mixtures was divided into two parts: one was to be used directly in the control diets; the other part was autoclaved for 30 minutes under 15 lb. pressure before being used in the experimental diets. This treatment had been found (Mader, Schroeder and Smith, '49) to decrease the nutritive value of protein.

The reducing sugar content of the commercial lactalbumin, extracted lactalbumin, and lactalbumin-sugar mixtures before and after autoclaving was determined by the Shaffer-Hartmann copper reduction iodometric method for determination of lactose in milk (Winton and Winton, '45). The results of these analyses (table 1) indicate the effectiveness of the extraction process, the accuracy of recombining this protein with these

[2] Obtained from the Borden Company.

sugars, and the decrease in reducing properties resulting from autoclaving the protein-sugar mixtures.

The basic diet used in this experiment was patterned after the purfied ration used by Orten and Smith ('37), except that vitamin capsules [3] were used in place of cod liver oil and yeast as a source of accessory food factors. The percentage composition of this basic diet was: lactalbumin 10.0, sucrose 44.0, white dextrin 12.8, hydrogenated cottonseed oil [4] 27.0, salt mixture [5] 3.5, bone ash 2.7.

The subjects in this experiment were three healthy adult female dogs weighing approximately 10 kg each. They were

TABLE 1

Analysis of sugar content

MATERIAL ANALYZED	SUGAR CONTENT	
	Unheated	Autoclaved
	millimoles %	*millimoles %*
Extracted lactalbumin with lactose	15.8	3.5
Extracted lactalbumin with maltose	16.2	6.3
Extracted lactalbumin with glucose	14.2	3.4
Extracted lactalbumin with fructose	15.6	5.2
Extracted lactalbumin with xylose	16.8	2.2
Extracted lactalbumin without sugar	1.2	1.2
Commercial lactalbumin	14.2	..

kept in standard metabolism cages with free access to water at all times. They were fed at a fixed hour each day and were weighed twice weekly.

The procedure followed in this experiment was adapted from the method described by Murlin and his associates ('41). In this method, the excretion of fecal and urinary nitrogen of a subject on a control diet containing a reference protein is compared with the urinary and fecal nitrogen excreted by the same organism when an experimental protein is sub-

[3] "ABDEC" capsules, kindly provided by Parke Davis and Company, Detroit, Michigan. Each capsule contained 18 mg of nitrogen.

[4] Crisco.

[5] Wesson.

stituted in the same diet. In this experiment, an unheated protein-sugar mixture (control) was used in the basic diet for three days, followed by a 4-day period in which the corresponding autoclaved protein-sugar mixture (experimental) was used. This sequence was repeated twice for each of the protein-sugar mixtures.

The urine was collected in jars containing sulfuric acid and complete collection for each period was insured by catheterization. The urine samples were diluted to a convenient volume and aliquots were analyzed for their nitrogen content. The feces were collected and covered with ethanol containing 1% sulfuric acid, and the samples were then dried on a steam cone. Nitrogen determinations were made upon aliquots of the ground dried feces, which were collected in carmine-marked periods. All analyses for nitrogen were made in replicate by the macro-Kjeldahl method.

Preliminary studies were conducted to determine the nitrogen and caloric requirement for each dog to maintain nitrogen equilibrium. In figure 1 are depicted the average balances obtained when the protein content and caloric intake for the dogs were varied. Since initial studies with a protein content of 10% in the diet resulted in a marked negative balance, the effects of increasing the protein content to 12½% and of increasing the individual ration of a 10% protein diet were studied. As a result of these preliminary studies, a diet containing 10% extracted lactalbumin was deemed satisfactory to maintain these dogs close to nitrogen balance when the caloric intake was sufficient to maintain body weight. Throughout the entire experiment, the dogs did not vary from their initial weights by more than 0.5 kg. As all the proteins used had the same nitrogen content, it was unnecessary to make isocaloric replacement of the proteins and sucrose to maintain a constant nitrogen content in the diet, except in one instance. The nitrogen content of the diets was $1.24 \pm 0.06\%$.

Complete nitrogen balance data on each dog for all control and experimental periods are presented in table 2. Figures 2 and 3 show in chronological sequence the averages of the nitro-

gen balances of the three dogs as they were alternately fed the heated and unheated protein-sugar mixtures. The changes in nitrogen balance varied in amount with each individual dog, but the direction of change was the same for every dog.

A modification of the method proposed by Murlin and associates ('41) was used to calculate the digestibility and biological values of the heated protein-sugar mixtures. The calculated digestibility and biological values are expressed

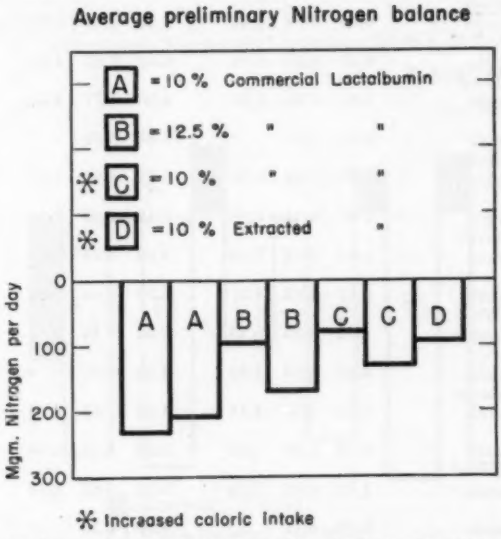
Average preliminary Nitrogen balance
A = 10 % Commercial Lactalbumin
B = 12.5 % " "
* C = 10 % " "
* D = 10 % Extracted "
0
100
200
300
Mgm. Nitrogen per day
A A B B C C D
* Increased caloric intake

Fig. 1 Preliminary nitrogen balance studies to estimate minimum nitrogen requirement of the dogs.

as percentages of the value of the control protein by assuming that the control protein nitrogen is completely absorbed. A third quantitative expression of the nutritional value of these proteins has been calculated by multiplying the biological value by the digestibility. This value, termed the "nutritive index" by Cahill, Schroeder and Smith ('44), more accurately expresses the over-all nutritive value of a protein than either the biological value or digestibility alone. In the calculation of these values for each autoclaved protein-sugar mixture, the

TABLE 2

Food, urinary and fecal nitrogen data

	TOTAL FOOD N [1]			TOTAL URINARY N			TOTAL FECAL N		
	Dog no.			Dog no.			Dog no.		
	1	2	3	1	2	3	1	2	3
	gm			*gm*			*gm*		
Extracted lactalbumin + lactose	4.62	4.62	3.69	4.92	4.90	3.44	0.82	0.58	0.85
Extracted lactalbumin + lactose, autoclaved	6.19	6.19	4.97	6.41	6.12	4.09	1.71	2.62	2.12
Extracted lactalbumin + lactose	6.43	6.43	5.21	5.27	6.25	4.20	0.86	0.68	0.86
Extracted lactalbumin + lactose, autoclaved	4.83	4.83	4.03	3.82	4.34	3.11	2.54	2.20	2.19
Extracted lactalbumin + maltose	4.84	4.84	3.93	4.20	4.89	2.90	0.23	0.28	0.43
Extracted lactalbumin + maltose, autoclaved	6.33	6.33	5.12	5.98	5.61	4.09	1.59	1.87	1.82
Extracted lactalbumin + maltose	4.84	4.84	3.93	4.20	4.77	3.14	0.50	0.51	0.73
Extracted lactalbumin + maltose, autoclaved	6.33	6.33	.. [2]	5.94	5.68	.. [2]	2.44	2.26	.. [2]
Extracted lactalbumin + glucose [3]	4.86	4.48	3.36	3.81	4.08	2.92	0.39	0.44	0.38
Extracted lactalbumin + glucose, autoclaved	5.87	5.42	3.78	5.42	5.45	3.64	2.47	2.47	2.05
Extracted lactalbumin + glucose	4.86	4.48	3.36	3.56	3.73	3.33	0.39	0.36	0.26
Extracted lactalbumin + glucose, autoclaved	5.87	5.42	4.07	5.50	5.40	3.84	2.86	1.76	2.15
Extracted lactalbumin + fructose	4.93	4.55	3.43	3.62	3.86	2.77	0.54	0.53	0.50
Extracted lactalbumin + fructose, autoclaved	6.48	5.98	4.50	4.88	5.05	3.78	3.18	2.13	2.24
Extracted lactalbumin + fructose	4.93	4.55	3.43	4.06	3.63	2.80	0.61	0.49	0.58
Extracted lactalbumin + fructose, autoclaved	6.48	5.98	4.50	5.01	5.50	3.64	2.80	2.26	1.90
Extracted lactalbumin + xylose	5.23	4.83	3.64	3.30	3.81	2.74	0.96	0.67	1.17
Extracted lactalbumin + xylose, autoclaved	6.66	6.14	.. [2]	4.80	4.30	.. [2]	2.17	2.51	.. [2]
Extracted lactalbumin + xylose	5.23	4.83	3.64	3.36	3.90	2.91	0.71	0.78	0.48
Extracted lactalbumin + xylose, autoclaved	6.66	6.14	4.62	5.84	5.43	3.80	2.98	2.69	2.06
Extracted lactalbumin without sugar	4.89	4.52	3.40	3.19	3.81	2.70	0.99	0.71	0.62
Extracted lactalbumin without sugar, autoclaved	6.46	5.97	.. [2]	4.62	4.74	.. [2]	1.88	1.53	.. [2]
Extracted lactalbumin without sugar	4.89	4.52	3.40	3.65	3.26	3.00	0.97	0.98	0.61
Extracted lactalbumin without sugar, autoclaved	6.46	.. [2]	4.49	4.28	.. [2]	3.39	2.18	.. [2]	1.72

[1] In most cases each unheated protein-sugar mixture was fed for a period of three days and each heated protein-sugar mixture was fed for a period of 4 days.

[2] Sample lost.

[3] Small dietary adjustments (caloric and nitrogen intake) made in this and succeeding periods to insure complete food consumption and no weight loss.

average of the data of the two experimental periods and the averages of the three control periods which separated and delimited them, have been used.

In table 3 are listed the calculated digestibilities for all of the heated protein-sugar mixtures and for the heated extracted lactalbumin without sugar. It is apparent from these

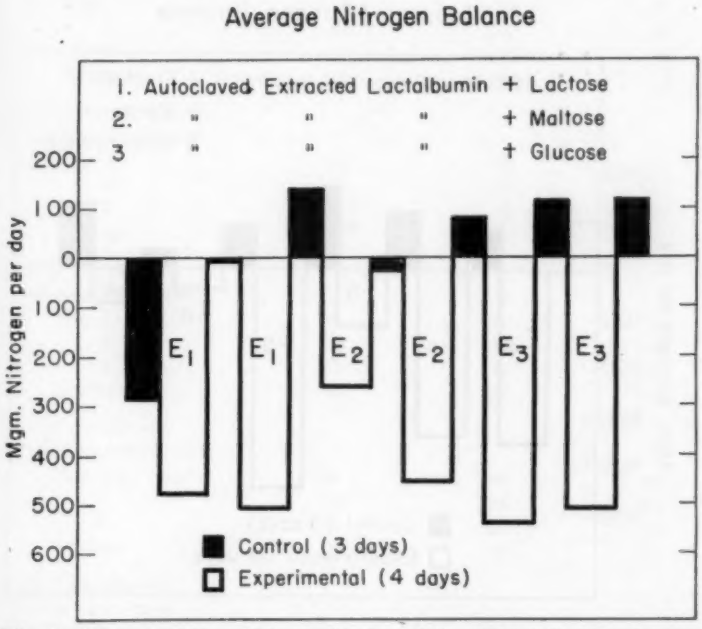

Fig. 2 Average nitrogen balances in dogs fed extracted lactalbumin-sugar mixtures (lactose, maltose, glucose).

data that the digestibility of the extracted lactalbumin combined with each of the reducing sugars had been severely impaired by autoclaving. Autoclaving the extracted lactalbumin which had not been combined with sugars caused a measurable decrease in the digestibility which, however, was strikingly augmented by autoclaving the protein in the presence of the various reducing sugars.

The average biological values of the autoclaved proteins are shown in table 3. On the basis of these data it is apparent that all of the nitrogen of the autoclaved proteins, whether combined with sugars or not, if absorbed, was utilized by the dog as well as the nitrogen absorbed during the control periods. Since the biological value of all of these proteins is uni-

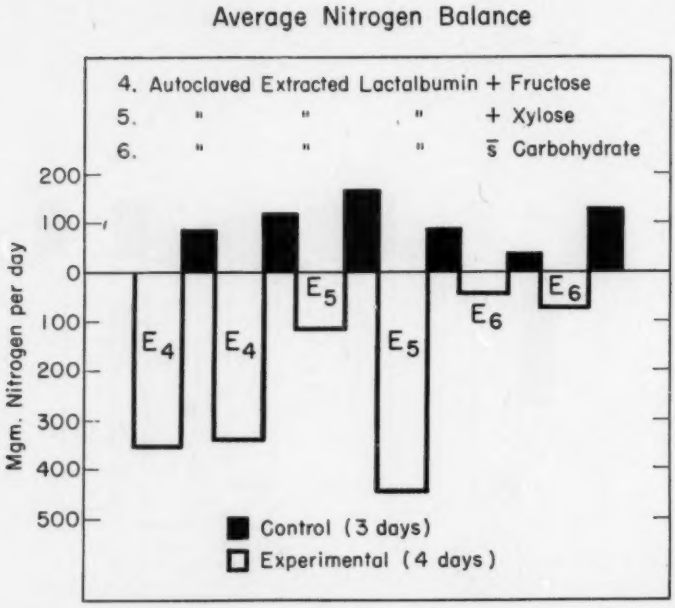

Fig. 3 Average nitrogen balances in dogs fed extracted lactalbumin-sugar mixtures (fructose, xylose).

formly high, the nutritive indices, given in table 3, reflect the same variation observed in the digestibility of these proteins. Whereas the nutritive index of the autoclaved extracted lactalbumin without sugar was 89% of that of the control protein, the nutritive indices of the autoclaved protein-sugar mixtures were reduced to from 64 to 78% of that of the control protein, a change due to the marked decrease in digestibility.

TABLE 3

Digestibility, biological value and nutritive indices of the autoclaved proteins

DIET PROTEIN	DIGESTIBILITY	BIOLOGICAL VALUE	NUTRITIVE INDEX
	%	%	%
Extracted lactalbumin + lactose, autoclaved			
Dog 1	72	98	71
2	65	107	70
3	67	102	68
Average	68	102	69
Extracted lactalbumin + maltose, autoclaved			
Dog 1	76	89	68
2	76	116	88
3	77	99	77
Average	77	101	78
Extracted lactalbumin + glucose, autoclaved			
Dog 1	65	85	55
2	72	94	68
3	62	112	69
Average	66	97	64
Extracted lactalbumin + fructose, autoclaved			
Dog 1	68	99	67
2	75	95	71
3	75	99	74
Average	73	98	71
Extracted lactalbumin + xylose, autoclaved			
Dog 1	79	82	65
2	73	106	77
3	77	97	75
Average	76	95	72
Extracted lactalbumin autoclaved			
Dog 1	86	99	85
2	93	99	92
3	80	111	89
Average	86	103	89

In vitro study

To verify further the impairment of digestibility resultant from interaction of the sugars and lactalbumin when heated, an *in vitro* digestion study was conducted upon these protein-sugar mixtures. For this purpose, the rate of digestion of the heated protein-sugar mixtures and unheated protein-sugar mixtures by pancreatin [6] was observed by formol titration, following the procedure outlined by Mecham and Olcott ('47).

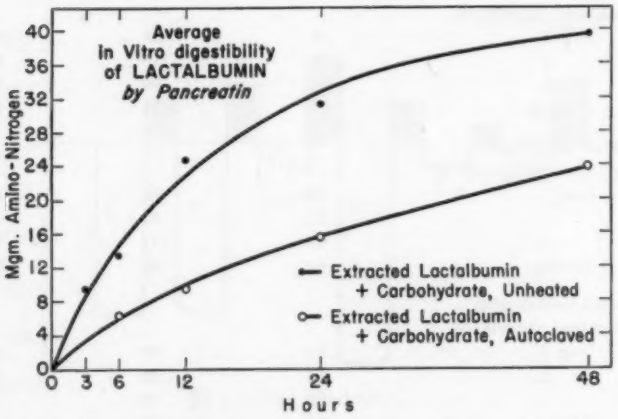

Fig. 4 *In vitro* digestibility of extracted lactalbumin-sugar mixtures, unheated and autoclaved, by pancreatin.

The average results of the digestion of the heated and unheated protein-sugar mixtures are graphed in figure 4. From these data it can readily be seen that the rate of digestion of the heated proteins by pancreatin was markedly decreased relative to that of the unheated control proteins. Thus, the impairment of digestibility observed in the *in vivo* study is also found in *in vitro* digestion of these proteins by pancreatin.

[6] Merck and Company, Inc.

It is evident from the data secured that there is no demonstrable difference between reducing sugars when used in equimolar concentrations under the experimental conditions employed. This observation serves as additional evidence that the Maillard reaction is dependent upon the reducing group of the carbohydrate.

DISCUSSION

The observations of Mader, Schroeder and Smith ('49) that the ability of extracted lactalbumin to maintain nitrogen balance in dogs is impaired by heating alone, and markedly impaired by heating in the presence of lactose, has been confirmed. The hypothesis that the reaction between reducing sugars and proteins may be responsible for the decreased nutritive value of heated proteins (Patton, Hill and Foreman, '48b; Henry and Kon, '50) is supported not only by this observation but by the fact that the same nutritive damage is observed when lactalbumin is heated with the same mole per cent of other reducing sugars.

Chemical studies of the browning reaction occurring between casein and various reducing sugars conducted by Lewis and Lea ('50) indicate that the aldopentoses react more rapidly than the aldohexoses. Disaccharides are even less reactive, while fructose was found to be least reactive of all. This order of variation in reactivity of the reducing sugars in the browning reaction was not indicated by significant statistical changes in our *in vivo* studies. The conditions employed in this experiment were considerably different from those used by Lewis and Lea. The heating conditions under which the present reaction was run were far more severe, while the concentration of sugar used was only about one-sixth that employed by the above authors. It is likely that, under the conditions employed, the reaction proceeded to such an extent with each of the sugars that variation in rate of reactivity would not be observed. Furthermore, the relative insensitivity of an *in vivo* study precludes the observation of slight changes.

On the basis of chemical studies of the mechanism of this reaction, Lea and Hannan ('50) concluded that it involves the interaction of the carbonyl groups of the sugar with free amino groups of the protein, and possibly other forms of binding of the sugar by the protein. They were unable to demonstrate the presence of glucose in the product of the interaction of glucose and casein, by acid or alkaline hydrolysis or by a specific glucose oxidase.

The mechanism of the nutritional effect which is produced by this reaction has been disputed. As was previously mentioned, Patton, Hill and Foreman ('48a) ascribed the deleterious effect of this reaction upon the nutritional value of a protein to extensive inactivation of certain essential amino acids. Evans and Butts ('49), following their *in vitro* investigation of the amino acid content of enzyme and acid hydrolysates of heated soybean oil meal and soybean oil meal heated with sucrose, suggested three types of inactivation of amino acids due to heating. They concluded that heating the protein alone resulted in the formation of linkages between certain of the amino acids which were resistant to digestion by pancreatin and erepsin. When the protein was heated with sucrose, amino acids with free amino groups were destroyed and certain others formed linkages with sucrose, which were resistant to enzymatic digestion *in vitro*. The *in vivo* studies of Weast, Groody and Morgan ('48) indicate that the decreased nutritional value of heated casein is due to poor absorption by the growing dog. Similarly, Mader, Schroeder and Smith ('49) attributed the nutritional damage produced by heating commercial lactalbumin to a decrease in digestibility. Using purified enzyme preparations, Lowry and Thiessen ('50) demonstrated that the product formed by the interaction of glucose and casein is resistant to tryptic digestion but is digested normally by pepsin *in vitro*. From the observations of Friedman and Kline ('50) it appears possible that in addition to decreased digestibility as a factor in lowering the nutritive value of heated protein-sugar mixtures, the reaction of the amino acids themselves with glucose may interfere with

their utilization. Heating a mixture of the essential amino acids per se, however, has been shown not to interfere with their utilization (Davis, Rizzo and Smith, '49).

In the present study a uniformly high biological value was observed when the protein-sugar mixtures were fed, indicating that what nitrogen was absorbed was as well utilized as the nitrogen of the control protein. Such results would not be expected if one or more amino acids had been extensively destroyed or otherwise made unavailable for mutual simultaneous supplementation. The nutritional damage observed in this study could be almost entirely attributed to a decrease in digestibility. Thus, in the browning reaction the formation of linkages which are not only resistant to enzyme digestion *in vitro,* but are also resistant to *in vivo* digestion, can account for the nutritional damage to this protein.

SUMMARY

The nutritional value of extracted lactalbumin, when autoclaved with equimolar concentrations of lactose, glucose, maltose, xylose and fructose, was significantly decreased; no distinction between these sugars could be demonstrated. When extracted lactalbumin was heated under similar conditions in the absence of reducing sugars, there resulted a decrease in nutritional value, but not so great a decrease as was observed when the reducing sugars were present. The decrease in nutritional value was attributed to a decreased digestibility. The biological value of that portion of the heated protein which was digested and absorbed was uniformly high.

LITERATURE CITED

CAHILL, W. M., L. J. SCHROEDER AND A. H. SMITH 1944 Digestibility and biological value of soybean protein in whole soybeans, soybean flour, and soybean milk. J. Nutrition, *28:* 209.

DAVIS, R. M., P. RIZZO AND A. H. SMITH 1949 The effect of heat on the nutritive value of lactalbumin. I. Growth on diets containing heated proteins. Ibid., *37:* 115.

EVANS, R. J., AND H. A. BUTTS 1949 Inactivation of amino acids by autoclaving. Science, *109:* 569.

FRIEDMAN, L., AND O. L. KLINE 1950 The relation of the amino-sugar reaction to the nutritive value of protein hydrolysates. J. Nutrition, *40:* 295.

HENRY, K. M., AND S. K. KON 1950 Effect of reaction with glucose on the nutritive value of casein. Biochim. et Biophys. Acta, *5:* 455.

HILL, E. G., AND A. R. PATTON 1947 The Maillard reaction in microbiological assay. Science, *105:* 481.

LEA, C. H., AND R. S. HANNAN 1950 Studies of the reaction between proteins and reducing sugars in the "dry" state. I. The effect of activity of water, of pH and of temperature on the primary reaction between casein and glucose. Biochim. et Biophys. Acta, *3:* 313.

LEWIS, V. M., AND C. H. LEA 1950 A note on the relative rates of reaction of several reducing sugars and sugar derivatives with casein. Ibid., *4:* 532.

LOWRY, J. R., AND R. THIESSEN, JR. 1950 Studies of the nutritive impairment of proteins heated with carbohydrates. II. In vitro digestion studies. Arch. Bochem., *25:* 148.

MADER, I. J., L. J. SCHROEDER AND A. H. SMITH 1949 The effect of carbohydrate on the nutritive value of heated lactalbumin. J. Nutrition, *39:* 341.

MECHAM, D. K., AND H. S. OLCOTT 1947 Effect of dry heat on proteins. Ind. Eng. Chem., *39:* 1023.

MURLIN, J. R., M. E. MARSHALL AND C. D. KOCHAKIAN 1941 Digestibility and biological value of whole wheat breads as compared with white bread. J. Nutrition, *22:* 573.

ORTEN, J. M., AND A. H. SMITH 1937 A study of certain metabolites and related compounds as precursors of endogenous citric acid. J. Biol. Chem., *117:* 555.

PATTON, A. R., AND E. G. HILL 1948 Inactivation of nutrients by heating with glucose. Science, *107:* 68.

PATTON, A. R., E. G. HILL AND E. M. FOREMAN 1948a Amino acid impairment in casein heated with glucose. Ibid., *107:* 623.

———— 1948b The effect of browning on the essential amino acid content of soy globulin. Ibid., *108:* 659.

RANSEY, R. J., P. H. TRACY AND H. A. RUEHE 1933 The use of corn sugar in the manufacture of sweetened condensed skim milk. J. Dairy Sci., *16:* 17.

ROSS, A. F. 1948 Deterioration of processed potatoes. Advances in Food Research. Vol. I, edited by E. M. Mrak and G. F. Stewart, Academic Press, Inc., New York, N. Y., pp. 257.

WEAST, E. O., M. GROODY AND A. F. MORGAN 1948 Utilization by dogs of the nitrogen of heated casein. Am. J. Physiol., *152:* 286.

WINTON, A. L., AND K. B. WINTON 1945 The Analysis of Foods. John Wiley and Sons, Inc., New York, N. Y., pp. 746.

BIOLOGIC UTILIZATIONS OF UNMODIFIED AND MODIFIED FOOD STARCHES

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TEN FIGURES

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Despite long-established recognition of differences in the histologic features of starch granules obtained from various plant materials, and more recent knowledge of the chemical heterogeneity of their starch-components, rather scant attention has been directed toward studies of the relative utilizations of starches as food for man and animals. The paucity of information on the relative food values of unmodified starches presumably stems from the facts that (1) most of the starch in human dietaries is consumed as modified starch, gelatinized or dextrinized; and (2) the widespread availability of cereal grains has largely allayed search for, or consideration of, other starch-rich feedstuffs for domestic livestock.

Scattered reports have appeared in the scientific literature of the past half-century with clear evidence that the human body manifests much greater efficiency in the digestion of certain unmodified starches — including those isolated from the cereal grains, cassava and taro — than in the case of others, such as those from the potato, arrowroot (genus *Maranta*) and canna (Fofanow, '11; Langworthy and Deuel, '20, '22; Langworthy and Merrill, '24). The starch in raw potato, in contrast to that in cooked potato, has also been

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noted to be poorly utilized by various species of livestock (Kennard, '36; Klooster and Card, '36; Gutteridge, '37; Halnan and Fermor, '42; Halnan, '44). Levy and Jacquot ('48) have reported unmodified potato starch to be poorly digested by normal young rats as compared with potato starch which has been ball-milled for three days, an observation which these workers interpret as indicating that the *in vivo* digestibility of raw starch depends upon the degree of polymerization of the starch molecules.

The investigations reported here were designed to throw further light upon (1) the nature of the physiologic mechanisms which limit the utilization of various unmodified starches while permitting almost complete utilization of others; and (2) certain basic attributes of starch granules associated with their different degrees of physiologic utilization. In pursuit of these objectives we have (1) investigated the physiologic utilizations of a variety of isolated starches with and without modification under strictly comparable conditions; (2) varied the management of the experimental animals (rats) in order to observe feeding conditions which would alter the degree of utilization of certain starches; and (3) conducted various *in vitro* experiments on starches which we believe may suggest some rational explanation of the divergences among unmodified starch granules from different sources as provisioners of food energy.

The sources of the starches investigated included wheat *(Triticum vulgare)*, rice *(Oryza sativa)*, maize *(Zea mays)*, cassava *(Manihot utilissima)*, arrowroot *(Maranta arundinacea)*, sago palm (an imported product from the Straits Settlements of Borneo, probably *Metroxylon rumphii* or *M. laeve)*, white potato *(Solanum tuberosum)*, and sweet potato *(Impomoea batatas)*.

EXPERIMENTAL

Descriptions of starches and starch products

Wheat starches included: (1) 4 commercial preparations made without use of chemicals except for the addition of

enough alkali to make the starch slurry neutral in reaction; (2) a commercial preparation, hydrochloric acid-modified to make a thin-boiling product; (3) a commercial preparation, oxidized with hypochlorite to achieve a thin-boiling product; (4) dry dextrinized wheat starch (torrefaction dextrins) prepared by heating thin layers of wheat starch in a rotary (2 r.p.m.) shelf-oven at 191°C. for 95 minutes with frequent stirring, which treatment was attended with loss, or very rapid fading, of the blue coloration on addition of iodine; (5) autoclaved wheat starch prepared by heating the starch moistened with 0.1% citric acid for 6 hours at 15 lb. steam pressure; (6) a gelatinized product prepared by heating an aqueous 20% starch slurry for 10 minutes at 15 lb. steam pressure, after which the product was spread in thin layers, dried overnight in a stream of air (at room temperature) and ground in a Wiley mill; and (7) a laboratory preparation made without the use of any chemicals.

Maize starches included: (1) a commercial preparation purchased in small retail packages; and (2) an unmodified commercial lot of waxy maize starch.

Rice and arrowroot starches included in each case one sample of an unmodified commercial product.

Sago starch included: (1) two lots of ordinary commercial type; (2) one of the commercial samples subsequent to ball-milling for two hours and 40 hours, respectively, wherein 125-gm batches along with 5 kg of one-half inch porcelain balls were added to a one-gallon container and rotated at 70 r.p.m.; (3) fecal residues from ingested commercial sago starch after partial purification to remove the major part of the non-starch fecal materials; and (4) commercial sago starch subsequent to one-hour treatments at room temperature of 20% slurries in which the aqueous phases consisted of 0.12, 0.16 and 0.20 M NaOH, respectively. The two commercial samples of this starch were associated with a small amount of brownish pigment along with other non-starch material from the pith or woody parts of the sago palm. The samples

of this starch contained on the anhydrous basis approximately 1.3% nitrogen, 0.15% fat, 0.42% fiber, 0.45% ash and 91% starch (by difference).

Cassava or "tapioca" starch included two lots of the usual commercial type prepared from cassava roots, no heat treatment being applied in the process.

Sweet potato starch included two lots of unmodified commercial products.

White potato starches included: (1) three lots of isolated commercial products; (2) a laboratory product prepared entirely without use of chemicals; (3) "Lintner's soluble" potato starch (Lintner, 1886) taken from a commercial lot manufactured for use in measurements of amylase activities; (4) a dry dextrinized product prepared by the same means described for torrefaction dextrins from wheat starch; (5) a vacuum-dried product (2.6% moisture) following 10 hours' refluxing with absolute alcohol and 44 hours' extraction with ether; (6) a sample of a commercial product ball-milled for two hours without prior treatment and a portion of the same sample ball-milled for two hours in the presence of absolute alcohol subsequent to 10 days' agitation and 6 months' standing, in the presence of absolute alcohol, at room temperature; (7) sample lots of an unmodified product separated by means of a laboratory air-classifier into a fraction concentrated in smaller granules (0.5% of the granules larger than 50 μ, 11.9% between 20 and 50 μ and 87.6% of 20-μ size or smaller), and a fraction concentrated in larger granules (15% of the granules 50 μ or more, 50% between 20 and 50 μ and 35% smaller than 20 μ); (8) a commercial product ball-milled for 40 hours as described for ball-milled sago starch; (9) gelatinized starch prepared by treating an aqueous 20% starch slurry precisely in the manner described for gelatinized wheat starch; (10) fecal residues from commercial potato starch, one with and one without partial purification to remove the major portion of the non-starch fecal materials, and (11) samples of a commercial product following one-hour treat-

ments at room temperature of 20% slurries with increasing concentration of alkali in the aqueous phases analogous to the products described under sago starch.

The physical characteristics of the granules in each of the above-mentioned starches, herein referred to as unmodified starches, were readily identifiable by microscopic examination, using both ordinary and polarized light; and, with the exception of the sago starch, very few of the starch samples revealed more than about 5% of disrupted or injured granules. The sago starch samples were estimated to consist of 20 to 25% of grossly and slightly injured granules and, as is characteristic of this kind of starch, many more granules showed a transverse fissure or a three-armed fissure radiating from the hilum. The sample of waxy maize starch yielded the characteristic reddish-brown coloration of amylopectin with iodine, while granules of all of the other unmodified starches stained a deep blue with the same reagent.

Animal-feeding techniques

All *in vivo* experimentation reported here, except as otherwise indicated, was initiated with weanling male rats of 45 to 60 gm body weight.

Each animal was housed in an individual cage having an elevated one-half inch mesh screen bottom in a laboratory maintained at a temperature of 23° to 25°C. and a relative humidity of between 40 and 60%. Each animal was weighed at least once each week and daily food records were kept for each. Continuing records were also kept of the development and progress of any outward signs of physiologic abnormalities among the animals.

Depending upon the specific objective in different instances, the feeding techniques employed were ad libitum feeding; equalized feeding of matched groups, in which the quantity of rations consumed daily was controlled by the individual animal in a given group that ate the least; or controlled, arbitrarily-restricted feeding of matched groups. Except for

variations described subsequently in the text, all of the diets used were constituted, in terms of dry weights of the ingredients, as follows:

	%		%
Starch	63.7	Salt mixture, modified, U.S.P. 1	4.0
Casein (commercial)	18.8	Cod liver oil, U.S.P. grade	1.0
Roughage material ("Ruffex")	2.0	Wheat germ oil	0.5
Cottonseed oil	9.4	Liver concentrate powder (1: 20)	0.6

Daily vitamin supplement per rat per day: thiamine hydrochloride 33 μg, riboflavin 67 μg, pyridoxine hydrochloride 20 μg, calcium pantothenate 100 μg, inositol 10 μg, *p*-aminobenzoic acid 500 μg, folic acid 20 μg, nicotinic acid 20 μg, biotin 0.2 μg and choline chloride 1 mg.

The 63.7% of anhydrous starch included in the above ration refers in the case of every kind of starch used, except sago starch, to the isolated product which contained, according to actual analyses, 98% or more of pure starch. The sago starch samples incorporated in this ration carried approximately 9% of non-starch (mostly nitrogenous) material.

In preliminary studies of the over-all or gross responses to different starches incorporated in the above ration at the 63.7% level, the ad libitum feeding technique was employed, thus allowing the animals to make such adjustments in food intakes as were physiologically induced or occasioned by variations in the utilization of the respective starches. For more quantitative comparisons of the relative utilizations of various unmodified and modified starches, one or the other of the matched-feeding techniques was employed. The relative biologic utilizations of the various unmodified and modified starches were assessed in terms of the following criteria: (1) their relative capacities to promote body weight increments in young rats when comprising the sole source of carbohydrate in the ration; (2) their respective coefficients of digestibility; (3) the respective average weights of dry feces excreted per gram of dry food ingested; and (4) notation of any aberrations in the gross appearance of the animals or their gastrointestinal tracts when they were sacrificed. Because of the massive accumulations of undigested starch in the ceca and colons of the rats when certain of

the starches investigated were consumed in the type-ration, the "net body weight gain" was computed for each animal by subtracting the initial gross live weight from the final gross live weight minus the weight of the posthumous contents of the gastrointestinal tract.

The digestibility coefficients for the various starches and starch products were determined in the usual manner from analyses of the food ingesta and fecal excretions (plus the posthumous contents of the gastrointestinal tracts) for moisture, ash, protein ($N \times 5.7$, 5.95, 6.25 or 6.38, whichever was judged to be most applicable for all or part of the dietary constituents), fat and crude fiber — the starch contents being computed "by difference." The fecal collections and records of food ingesta, except where specifically indicated, covered the entire span of the experimental periods.

RESULTS

As is evidenced by the data in table 1, the various unmodified starches investigated may be grouped roughly into three categories as regards their relative utilizations; viz., those approaching almost complete (98%) digestibility and in the highest degree contributive to the support of body weight gains — wheat, maize, rice and cassava; those showing decidedly low coefficients of digestibility and limited by reason thereof as regards support of the body weight increments of young animals — arrowroot, sago and white potato; and sweet potato starch, intermediate as regards digestibility and assimilability. No outward signs of physical abnormality were noted among any of the animals on unmodified cereal or cassava starches. The acid-modified and the oxidized wheat starches elicited responses indistinguishable from those obtained with unmodified wheat starch. It is obvious, on inspection of the data in table 1, that some groups of rats on the type-ration containing unmodified potato and sago starches, respectively, consumed rather extraordinary amounts of food as if, teleologically speaking, to compensate for the decidedly poor digestibilities of these two starches.

TABLE 1

Comparisons of relative utilizations of various unmodified and modified starches as determined by ad libitum feeding of diets containing 63.7% (anhydrous basis) of the starch products specified. (Animals 45–60 gm in initial weight; feeding periods, 28 days unless otherwise specified in column 1)

STARCH COMPONENT OF DIET	EXPT'L. ANIMALS [1]	TOTAL DRY FOOD EATEN	NET BODY WEIGHT GAIN [2]		AVERAGE FINAL BODY LENGTH	DRY FECES PER GRAM DRY FOOD EATEN	STARCH, COEFF. OF DIGESTIB.
			Total	Per gram dry food			
	no.	gm S.D.	gm S.D.	gm S.D.	cm	gm	%
Wheat 1	6	283 ± 21	127 ± 11.7	0.45 ± 0.022	19.9	0.05	98.4
Wheat 1	6	310 ± 4	126 ± 9.9	0.41 ± 0.022	20.1	0.05	97.8
Wheat 2 acid-modified	6	302 ± 23	131 ± 7.7	0.43 ± 0.021	20.4	0.05	98.3
Wheat 3 oxidized	6	304 ± 14	126 ± 6.8	0.41 ± 0.011	20.2	0.05	98.4
Maize 1	6	313 ± 17	121 ± 18.4	0.39 ± 0.043	19.8	0.05	97.7
Maize, waxy, 1	6	312 ± 11	130 ± 5.2	0.42 ± 0.006	19.9	0.05	98.3
Rice 1	6	322 ± 20	130 ± 11.5	0.40 ± 0.015	20.2	0.06	97.5
Cassava 1	6	307 ± 40	110 ± 27.2	0.36 ± 0.059	19.6	0.05	98.0
Cassava 2	6	326 ± 33	135 ± 16.5	0.42 ± 0.021	20.4	0.05	97.7
Sweet potato 1	6	301 ± 20	121 ± 14.7	0.40 ± 0.025	19.9	0.07	96.6
Sweet potato 2	6	281 ± 25	97 ± 6.4	0.35 ± 0.025	19.3	0.09	95.4
Arrowroot 1	6	293 ± 31	91 ± 7.2	0.31 ± 0.021	19.1	0.18	79.9
Sago palm 1	8	364 ± 36	114 ± 5.5	0.31 ± 0.016	20.0	...	.
Sago palm 1	6	342 ± 59	109 ± 21.6	0.32 ± 0.013	19.8	0.26	65.3
Sago palm 1 ball-milled 2 hr.	6	349 ± 31	135 ± 18.2	0.39 ± 0.025	20.0	0.09	95.1
Sago palm 1 ball-milled 40 hr.	6	334 ± 20	127 ± 10.8	0.38 ± 0.024	19.9	0.06	96.5
White potato 1	5(1d)	337 ± 28	106 ± 13.0	0.31 ± 0.028	19.7	...	...
White potato 2, lab. product, no chemical treatment	4	350 ± 88	95 ± 20.6	0.27 ± 0.021	19.5	0.35 [3]	50.9 [3]
White potato 1 ball-milled 40 hr.	6	278 ± 28	111 ± 11.7	0.40 ± 0.014	19.5	0.05	98.2
White potato 3, Lintner's soluble	8	384 ± 44	107 ± 13.8	0.28 ± 0.027	19.5	0.37	51.0
White potato 4; 7-day feeding period	12	48 ± 10	9 ± 4.8	0.17 ± 0.076	14.4	0.43	42.7
White potato 4; 14-day feeding period	12	123 ± 24	35 ± 9.0	0.28 ± 0.041	16.5	0.38	49.8
White potato 4; 21-day feeding period	12	221 ± 29	72 ± 8.5	0.32 ± 0.020	18.4	0.35	53.8
White potato 4; 28-day feeding period	9(3d)	328 ± 55	100 ± 12.6	0.31 ± 0.021	19.7	0.32	59.2

[1] Parenthetical figures in this column indicate number of animals that did not survive the experimental period, all records on which were excluded in computation of the data presented in columns 3 to 8 inclusive.

[2] Net gain = final gross live weight (posthumous contents of G.I. tract + initial gross live weight).

[3] The posthumous contents of the G.I. tracts were not collected for this group of animals, hence not taken into account in this computation.

The data assembled in the last grouping of table 1 show that, with prolongation of the feeding period, young animals appeared to exhibit a gradually and continuously increasing coefficient of digestibility for unmodified potato starch. However, as is shown in several groupings of data in tables 1 and 2, it is obvious that animals subsisting on diets containing 63.7% of unmodified potato starch frequently did not survive relatively short feeding periods. Moreover, it was found that the gradual increase in digestibility coefficients for unmodified potato starch, noted among young animals with prolongation of the feeding period, were not to be explained on the basis of increasing age or stage of development. Two groups of 6 male animals each from our stock colony, when placed at 90 days of age on the type-ration carrying 63.7% of unmodified potato starch, were observed, under ad libitum feeding conditions, to digest on the average 55.6% of the starch ingested (average, 161 gm per rat) over a period of two weeks and 73.5% of that consumed (average, 302 gm per rat) over a period of 4 weeks. Similar groups comprised of 6 adult male animals, one year of age and weighing over 400 gm each, when placed on the type-ration containing 63.7% of unmodified potato starch, were observed to digest on the average 58.0% of the starch ingested (average, 144 gm per rat) over a period of two weeks and 64.6% of that consumed (average, 293 gm per rat) over a 4-week period.

In the light of the much higher food consumption of older animals it is obvious that they digested larger amounts of unmodified potato starch than did animals only two to 4 weeks beyond weanling age. It would seem, then, that whatever factor was involved in limiting the digestibility of unmodified potato starch is concerned with a digestive capacity more or less corresponding to normal over-all requirements for food energy.

In the case of animals subsisting on the type-ration containing unmodified potato, sago and arrowroot starches, the question arose of whether the coefficients of digestibility observed for these starches were inordinately depressed by

reason of the accompanying large bulk of undigested material's having hastened the passage of food through the small intestines, and thus having created something of a vicious circle. Groups of 7 animals on stock ration until 33 days of age were starved overnight and then transferred to carmine-marked type-rations containing 63.7% of unmodified potato, sweet potato and wheat starches, respectively, and were observed to begin excretion of the marker in 5.82 $\pm$ 0.94 (S.D.) hours, 16.67 $\pm$ 4.58 hours and 15.31 $\pm$ 5.67 hours, respectively. After one week or 6 weeks on these same type-rations, added carmine marker began to be excreted in 10 to 12 hours, irrespective of the kind of starch in the ration. Obviously, then, any accelerated rate of passage of unmodified potato starch relative to that of unmodified sweet potato or wheat starch was limited to the first few days of the experimental feeding period. We observed no significant differences in average daily water intakes or in percentages of moisture in the cecal contents among comparable groups of animals on the type-ration containing 63.7% of unmodified potato and wheat starches.

On the basis of the experiments just described, the possibility suggests itself that what appeared to be a gradually increasing coefficient of digestibility for unmodified potato starch with prolongation of the feeding period may have involved nothing more than an averaging out of the effects of the abnormally low coefficient of digestibility for the first few days. As something of a corollary to this proposition, it was observed that a group of 12 weanling rats transferred immediately to the type-ration containing 63.7% of unmodified potato starch, and three other groups of 12 each which were transferred to this same ration after one, two and three weeks on our stock ration, showed at the end of one week on the type-ration average digestibility coefficients for unmodified potato starch no. 4 of 43.0, 44.1, 50.3 and 46.7%, respectively. In the light of these two complementary lines of observation we are inclined to regard any *seeming* increases in digestive capacity for unmodified potato starch,

subsequent to the first few days on the type-ration containing 63.7% of this starch, as more likely to be an artifact (inherent in the usual method of computation) than a reality.

Langworthy and Deuel ('22) noted in their studies with human subjects that those unmodified starches found by them to be of relatively low digestibility were also the ones composed of granules of relatively large size, such as potato, arrowroot and canna starches. These authors questioned whether this was accidental or was occasioned by the larger surface areas or attributable to the thicker "cellulose" covering of the larger starch granules. As we have already pointed out, unmodified sweet potato starch, whose range in granule size is included within the limits of the sizes of wheat, maize and cassava starch granules, was repeatedly observed by us to be significantly less digestible than the unmodified cereal or cassava starches. In addition, entirely comparable feeding tests conducted on potato starch granules from a single lot of starch (see first grouping of data in table 2) showed no significant differences among the digestibilities of the fraction concentrated in smaller granules, the fraction concentrated in larger granules, and a sample of the original assortment of unfractionated granules. Photomicrographs of the original sample of potato starch and of the smaller granule fraction, respectively, used in these experiments are shown in plate 1 along with photomicrographs of fecal residues obtained from animals fed these two products at the 63.7% level in the type-ration.

Since a portion of the granules in every lot of unmodified potato, sago and arrowroot starches was digested, while another portion was excreted in large measure as intact granules in the feces, it might be supposed that some considerable lack of homogeneity existed among the granules from each of these sources as regards susceptibility to *in vivo* digestion. Inspection of the second grouping of data in table 2 suggests that the undigested potato starch granules excreted by one set of animals and incorporated as dried fecal material, in

TABLE 2

Comparative physiologic responses to diets containing 63.7% of various unmodified starches and equivalent levels of treated or recovered starch products, as determined by controlled feeding techniques. (Animals used were young male rats, 45–60 gm initial weight)

CARBOHYDRATE COMPONENT OF DIET	EXPT'L. ANIMALS [1]	EXPT'L. PERIOD	TOTAL DRY FOOD EATEN	NET BODY WEIGHT GAIN [2]				AVERAGE FINAL BODY LENGTH	DRY FECES PER GRAM DRY FOOD EATEN		CARBOHYDRATE COEFFICIENT OF DIGESTIBILITY	
				Total		Per gram of dry food						
	no.	days	gm	gm	S.D.	gm	S.D.	cm	gm	S.D.	%	S.D.
White potato starch 1, unfractionated	4	14	125	36 ±	1.5	0.29 ± 0.019		16.3	0.40 ± 0.034		46.2 ±	5.90
White potato starch 1, large gran. fraction	4	14	128	37 ±	7.6	0.29 ± 0.037		16.3	0.35 ± 0.037		53.2 ±	6.99
White potato starch 1, small gran. fraction	4	14	128	37 ±	5.8	0.29 ± 0.030		16.4	0.39 ± 0.017		47.0 ±	2.90
White potato starch 4	5(1d)	10	68 [3]	14 ±	9.0	0.20 ± 0.109		14.0	0.38	....	50.5	....
White potato starch 4, fed as feces	5(1e)	10	68 [3]	10 ±	4.8	0.14 ± 0.071		13.9	0.32	....	65.4	....
White potato starch 4	6	7	51 [3]	11 ±	5.6	0.20 ± 0.098		14.1	0.45 ± 0.021		38.9 ±	3.48
White potato starch 4, recovered from feces	6	7	51 [3]	10 ±	3.4	0.19 ± 0.050		14.4	0.42 ± 0.062		46.7 ±	10.51
White potato starch 1, ball-milled 2 hr.	4	21	200	77 ±	2.3	0.39 ± 0.015		18.2	0.12 ± 0.012		90.5 ±	2.07
White potato starch 1, alcohol-extracted and ball-milled 2 hr.	4	21	200	74 ±	4.4	0.37 ± 0.027		18.2	0.14 ± 0.014		87.0 ±	2.41
Wheat starch 1	8	21	212	93 ±	9.3	0.44 ± 0.022		18.9	0.05 ± 0.003		98.0 ±	0.24
Wheat starch 1 dextrinized (dry heat)	8	21	212	88 ±	8.8	0.42 ± 0.017		18.7	0.05 ± 0.003		97.7 ±	0.21
Wheat starch 1 autoclaved (acid-medium)	8	21	212	91 ±	7.4	0.43 ± 0.013		18.9	0.09 ± 0.005		94.3 ±	2.10
White potato starch 1	8	28	296	89 ±	13.2	0.30 ± 0.039		18.9	0.29	....	68.5	....
White potato starch 1 dextrinized (dry heat)	8	28	299	109 ±	9.5	0.36 ± 0.024		19.9	0.11	....	91.4	....
Wheat starch 6 gelatinized	10	7	42	17 ±	2.3	0.41 ± 0.055		14.4	0.07	....	95.8	....
White potato starch 5 gelatinized	10	7	42	17 ±	1.5	0.39 ± 0.036		14.5	0.07	....	95.5	....
White potato starch 5	8	7	44	5 ±	5.2	0.12 ± 0.118		14.5	0.40 ± 0.036		45.6	....
White potato starch 5 (0.12 M NaOH-treated)	5(3e)	7	44	7 ±	3.4	0.15 ± 0.078		14.7	0.40 ± 0.044		45.3	....
White potato starch 5 (0.16 M NaOH-treated)	7(1e)	7	44	7 ±	6.5	0.17 ± 0.127		14.6	0.36 ± 0.024		52.9	....
White potato starch 5 (0.20 M NaOH-treated)	6(2e)	7	44	16 ±	4.2	0.36 ± 0.096		15.1	0.22 ± 0.004		73.5	....
Sago palm starch 2	7(1e)	7	44	12 ±	4.5	0.28 ± 0.103		14.9	0.24 ± 0.018		68.7	....
Sago palm starch 2 (0.12 M NaOH-treated)	8	7	44	12 ±	3.8	0.27 ± 0.086		14.8	0.25 ± 0.021		67.6	....
Sago palm starch 2 (0.16 M NaOH-treated)	7(1e)	7	44	13 ±	4.6	0.29 ± 0.104		14.9	0.19 ± 0.029		76.7	....
Sago palm starch 2 (0.20 M NaOH-treated)	6(2e)	7	44	18 ±	3.4	0.42 ± 0.078		15.2	0.10 ± 0.010		91.9	....

[1] Parenthetical figures in this column followed by "d" indicate numbers of animals which died prior to conclusion of test-period; those followed by "e" indicate numbers of animals which did not consume prescribed amounts of the diet. Only those animals not included in parentheses were included in averages shown in other columns of data.

[2] Net gain = final gross live-weight (posthumous contents of G.I. tract + initial gross live-weight).

[3] In order that this diet should provide the same level of protein, starch and fiber as the diet with which it was pair-fed, it was necessary to make it a slight modification of the type-ration. This necessity arose from the fact that undigested protein and fiber were associated with undigested starch in the feces.

the amount required to provide 63.7% of dry starch in the ration of another set of animals, may have been slightly more digestible than the original potato starch fed in identical quantity. However, microscopic examination of the feces from rats on a diet containing 63.7% of unmodified potato starch, as is indicated in figure 2, plate 1, revealed that an appreciable proportion of undigested starch granules had been disrupted or eroded during passage through the digestive tract. Removal of most of these injured granules and much of the non-starch food residue through partial purification of the fecal residues yielded a product possessing a digestibility coefficient not significantly different from that of the original, unmodified granules, as evidenced by data presented in the third grouping of data in table 2. Analogous sets of data (not recorded here) were obtained in feeding trials with sago starch recovered from the feces. It may be concluded, therefore, that lack of homogeneity among granules of a given sample of potato or sago starch offers no possibility of explaining the low assimilabilities of these starches.

Having at this stage been led to consider that the prime physiologic factor which limits the utilization of various unmodified, non-cereal, starches may well be the lack of a sufficiently powerful digestive mechanism, it became of interest to investigate the nature of the modifications of these starches which enhanced their physiologic utilization.

As is evidenced by data presented in table 2, gelatinization and dextrinization of potato starch are attended with marked increases in digestibility and assimilability of the modified products, whereas in the case of wheat starch these treatments are of very little consequence. Guerrant and Dutcher ('34), Guerrant et al. ('37) and Mannering et al. ('44) reported observations indicative of some slight lowering in the absorption of the end products of digestion of dextrinized cornstarch and commercial dextrins as compared with isolated raw cornstarch. An analogous situation was found here for aqueous acid-dextrinized wheat starch, but the digesti-

bility and assimilability of dry-dextrinized wheat starch were not found to be significantly different (statistically) from those of untreated wheat starch. In contrast to this, the torrefaction dextrins from potato starch, prepared under conditions identical with those prevailing with respect to the dextrins of wheat starch, were found to be only about 91% digestible. In all likelihood the digestibilities of both aqueous, acid-dextrinized and dry-dextrinized starches vary with the source of starch and with the detailed conditions of their preparation, especially if these conditions invoke some "browning" or carmelization. As is shown by data in table 2, completely gelatinized wheat and potato starches are equally digestible and equally effective and efficient in supporting growth in young animals.

On the basis of data not included here because of space limitations it was shown that a step-wise dilution of unmodified potato starch in the type-ration with unmodified wheat starch or with dextrose, while the potential heat of combustion in the food consumed was held constant, was attended with consistent step-wise increases in all criteria indicative of higher assimilability of the potato starch components of the diets. However, in terms of the absolute quantities of potato starch digested, there was a step-wise decrease as the proportion of potato starch in the ration was reduced by dilution with unmodified wheat starch or dextrose. These observations on animals not only confirm but extend the less comprehensive observations noted in studies on human adults by Langworthy and Deuel ('20, '22) and Langworthy and Merrill ('24).

Prolonged refluxing of potato starch (previously dried to a moisture content of 2.6%) with absolute alcohol, followed by extraction with ether, was entirely without effect upon the utilization of the resultant product. As is shown by data in table 2, prolonged refluxing of dehydrated potato starch (2.6% moisture) with absolute alcohol followed by two hours of ball-milling in the presence of absolute alcohol did not in the least enhance the digestibility of the product over that

of a sample from the same lot which was ball-milled for two hours without any prior treatment.

In the 8th and 9th sets of data in table 2 are shown various physiologic criteria indicative of the relative degrees of assimilation of potato and sago starch granules subsequent to their treatment with different concentrations of aqueous NaOH. Application of the "t" test to these two sets of data did not establish statistically significant improvement in assimilability by reason of the alkali treatments except when the concentration of alkali used was 0.20 M. The marked swelling or hydration of the granules in 0.20 M NaOH, just as in the case of injury of the physical structures of a considerable proportion of the granules with two or more hours of ball-milling, is attended with a significant increase in digestibility of those starches originally of low digestibility. It is also obvious that the digestibilty of sago starch granules treated with 0.20 M NaOH approached that of the untreated cereal starches, while potato starch (originally more poorly digested than the sago starch) treated in the same manner attained a digestibility of only about 74%. In view of these experiments, the normal alkaline gradients of the digestive juices of the small intestine would appear not to be concerned at all with the digestibility coefficients observed for unmodified potato and sago starches.

Inspection of the data in table 1 relative to ball-milled sago starch and of the data in tables 1 and 2 relative to ball-milled potato starch will show clearly that ball-milling for as short a period as two hours — which is, according to Lampitt et al. ('47, '48), far short of the time required to depolymerize most of the molecules of potato starch — sufficed to raise the digestibilities of these modified starches to values only slightly less than did 40 hours of ball-milling, and only slightly less than the digestibilities observed for cereal grain and cassava starches.

In vitro digestibility experiments employing two-hour incubation periods at 38°C. of mixtures comprised of 2 gm U.S.P. pancreatin, and 0.5 gm NaCl, along with different

amounts of raw, solid starch suspended in enough phosphate buffer (pH 7.0) to provide a total aqueous phase of 50 ml, have shown: (1) that the structural organizations of the intact wheat starch granules are attacked by enzymatically active pancreatin (see fig. 10, plate 2), with practically all of the granules shattered when 1% of unmodified wheat starch is included in the mixture, and markedly decreasing proportions shattered as the percentage of the same starch in the mixture is increased from 1 to 5 and 10%; (2) that the production of reducing sugars in the mixtures increased (maltose equivalent to 525, 1,210 and 1,490 mg after subtraction of appropriate blanks for reducing powers of the pancreatin and of the starch substrates in the absence of pancreatin) as the percentages of the unmodified wheat starch therein were increased from 1 to 5 and 10%; (3) that both wheat and potato starch granules which had been ball-milled for two hours yielded increasing amounts of total reducing sugars (and graded decreases in maltose equivalents *per gram* of starch substrate) as the percentages of these starch substrates in the mixtures were increased from 1 to 5 and 10%; (4) that wheat and potato starches modified by two hours of ball-milling yielded very similar quantities of reducing sugars from identical amounts of these respective substrates included in the digestion mixtures; while (5) so few unmodified potato starch granules were affected by digestion with pancreatin as to make it doubtful whether any granules were attacked which had not been subject to prior injury (see fig. 8, plate 2).

We have also noted repeatedly that the addition of 0.25 to 1 gm of whole powdered ox-gall to our pancreatin digest mixtures which included unmodified wheat and potato starch, respectively, appeared to increase the proportion of granules showing evidence of disrupted physical structures. The unmodified wheat and potato starches employed in our *in vitro* digestion experiments were laboratory preparations, estimated by microscopic counts to carry less than 0.5% of injured granules. These *in vitro* experiments, therefore, proved indeter-

minate with regard to the question of whether the outermost layers of unmodified wheat and potato starch granules consist of modifications of starch substance or of integuments of non-starch material; but, whichever they may be, those of wheat are far more susceptible to structural disorganization and formation of reducing sugars by action of U.S.P. pancreatin than are intact potato starch granules. Resolution of this question could presumably be achieved only by experimentation with more highly purified enzyme preparations than were available to us.

Our experimental work considered *in toto* suggests that the relatively low digestibilities of such unmodified starches as those from the potato, sago palm and arrowroot are entirely attributable to some firmer degree of hydrogen-bonding of the molecules (or some other inherent characteristic) of the outermost layer or layers of the granules than is the case with starch granules from the cereal grains, cassava root and, in lesser degree, from the sweet potato, which makes the former granules more resistant to attack by amylase. From a physiologic standpoint the low digestibilities of unmodified potato, sago and arrowroot starches relative to cereal and cassava starches must, seemingly, be due to insufficient concentration of amylase in the digestive juices, or insufficiency of some other agent in the digestive medium which is required to enhance the activity of amylase or to modify these starches.

As a matter of some general interest, it was also noted throughout the experiments herein reported, whether the food intakes of the animals were restricted or unrestricted, that the apparent digestibility coefficients for fat were consistently 97% or greater, while the apparent digestibility coefficients for protein (from around 80 to 97%) varied directly with the digestibility of the starch component.

DISCUSSION

In general there would appear to be some correlation between the relatively low digestibility coefficients observed

for various unmodified non-cereal starches (sweet potato, arrowroot, sago and potato) and the continuous storage of these granules in plant organs possessing high water content (roughly 70% or higher) until time of harvest or until the full maturation of the plant organ in which the starch is stored. Could the outermost layers of starch granules represent gradations in hydrogen-bonding corresponding roughly with the moisture content in which the granules were continuously or finally stored in the intact plant organs? A search of the literature for data on the moisture content of thoroughly fresh but mature samples of plant organs carrying the different kinds of non-cereal starches investigated by us would suggest this possibility, as revealed in reports by Adriano et al. ('32), Raymond et al. ('41), Yoshimura and Sagava ('18) and Chatfield and Adams ('40) although data in this last reference undoubtedly include, in the averages, products analyzed at considerable intervals after harvesting. Starch deposited in cereal grains, of course, begins in a high moisture environment which steadily decreases until a moisture content normally less than 15% is achieved in the fully-ripened grains.

SUMMARY

Investigations of unmodified starches, including those from cereal grains (wheat, rice, maize and waxy maize), from aerial stems (sago palm) and from plant roots and subterranean stems (arrowroot, cassava, sweet potato and white potato) have shown these, under thoroughly comparable conditions of feeding, to possess wide differences in physiologic assimilability.

Unmodified starches isolated from cereal grains and cassava roots evidenced very high degrees of assimilability coincident with roughly 98% digestibility; those from arrowroot, white potato and sago palm evidenced decidedly low degrees of digestibility which varied inversely with the intake or dietary level of these starches; and that from the sweet potato occupied something of an intermediate position.

Viewed from the standpoint of physiologic processes, the data herein presented suggest that the relatively low digestibilities of unmodified sweet potato, potato, arrowroot and sago starches were occasioned by an insufficient amount or concentration of amylase, or of some agent in the digestive juices which is required to modify these starches prior to attack by amylase.

Viewed from the standpoint of the digestion-resistant properties of unmodified sweet potato, potato, arrowroot and sago starches, it would appear that this property resides consistently and exclusively in the outermost layers of the organized granules and, therefore, according to modern concepts, is associated with the hydrogen-bonding of these structures or with a granule integument of non-starch substance. Conditions which increase the digestibility of these starches include various modifications which produce obvious hydration of the granules, distinct changes in chemical nature, or disruption of the organized granule structures. Despite the *frequent* association between large-sized starch granules and digestion-resistant properties, the evidence presented here suggests that this is merely coincidental and *not exclusive*. It has also been shown that lack of homogeneity as regards digestion-resistant properties among granules of such unmodified starches as those of the potato or the sago palm offers no basis for explaining the low digestibility coefficients of these starches. There is some suggestion that, teleologically speaking, the digestion-resistant properties of starch granules from certain sources exist as a protective factor during storage *in situ* under conditions of high moisture.

According to the observations made here, unmodified wheat starch granules which show no signs of injury are quite susceptible to digestion *in vitro* by U.S.P. pancreatin preparations, and very likely the same is true of cereal starches in general.

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LITERATURE CITED

ADRIANO, F. T., H. T. RAMOS AND L. A. YNALVEZ 1932 The proximate chemical analysis of Philippine foods and feeding stuffs. III. Philippine Agr., *20:* 530–534.

CHATFIELD, C., AND G. ADAMS 1940 Proximate composition of American food materials. U. S. Dept. Agr. Circ. 549.

FOFANOW, L. 1911 Digestion and absorption of several varieties of raw starch in the normal and diseased gastrointestinal tract. Zschr. klin. Med., *71:* 257–270.

GUERRANT, N. B., AND R. A. DUTCHER 1934 Some effects of the composition of the diet on the vitamin B and the vitamin G requirement of the growing rat. J. Nutrition, *8:* 397–420.

GUERRANT, N. B., R. A. DUTCHER AND R. A. BROWN 1937 Further studies concerning the formation of the B-vitamins in the digestive tract of the rat. Ibid., *13:* 305–315.

GUTTERIDGE, H. S. 1937 Methods and rations for fattening poultry. I. Sci. Agr., *17:* 6–10.

HALNAN, E. T. 1944 Digestibility trials with poultry. XI. The digestibility and metabolizable energy of raw and cooked potatoes, potato flakes, dried potato slices and dried potato shreds. J. Agr. Sci., *34:* 139–154.

HALNAN, E. T., AND C. E. FERMOR 1942 Surplus potatoes in poultry feeding. Agriculture (England), *49:* 175–179.

KENNARD, D. C. 1936 Potatoes for chickens. Ohio Agr. Exp. Sta. Bimonthly Bull., *21:* 111–113.

KLOOSTER, M. J., AND C. G. CARD 1936 Potatoes as a feed for chickens. Mich. State Coll. Agr. Exp. Sta. Quart. Bull., *19:* 104–108.

LAMPITT, L. H., C. H. F. FULLER AND N. GOLDENBERG 1947 The fractionation of potato starch. I. The process of grinding. J. Soc. Chem. Ind., *66:* 417–421.

———— 1948 The fractionation of potato starch. II. The reducing powers, mean molecular weights and solubilities of the fractions. Ibid., *67:* 38–40.

LANGWORTHY, C. F., AND H. J. DEUEL, JR. 1920 Digestibility of raw corn, potato and wheat starches. J. Biol. Chem., *42:* 27–40.

———— 1922 Digestibility of raw rice, arrowroot, canna, cassava, taro, tree-fern and potato starches. Ibid., *52:* 251–261.

LANGWORTHY, C. F., AND A. T. MERRILL 1924 Digestibility of raw starches and carbohydrates. U. S. Dept. Agr. Bull. 1213, pp. 1–5.

LEVY, J., AND R. JACQUOT 1948 Polymérisation et digestibilité: influence du broyage sur la digestibilité de la fécule. Compt. rend., *227:* 371–373.

LINTNER, C. J. 1886 Studien uber Diastase. J. prakt. Chem., *34:* 378–394.

MANNERING, G. J., D. ORSINI AND C. A. ELVEHJEM 1944 Effect of the composition of the diet on the riboflavin requirement of the rat. J. Nutrition, *28:* 141–156.

RAYMOND, W. D., F. I. C. JOJO AND Z. NICODEMUS 1941 The nutritive value of some Tanganyika foods. II. Cassava. E. African Agr. J., *6:* 154–159.

YOSHIMURA, K., AND N. SAGAVA 1918 The chemical constituents in *Cycas revolute* Thunb I. Tokyo Kwagaku Kwaishi (J. Tokyo Chem. Soc.). *39:* 1116–1121.

PLATE 1

EXPLANATION OF FIGURES

Photomicrographs (× 100) showing:

1 Unmodified potato starch sample 1.

2 Fecal residue from a representative animal fed the type-ration carrying 63.7% of unmodified potato starch 1.

3 Small granule fraction of unmodified potato starch 1 separated with laboratory air-classifier.

4 Fecal residue from an animal fed the small granule fraction of unmodified potato starch depicted in (3) above.

5 Potato starch 1 after 40 hours of ball-milling.

6 Fecal residue from a representative animal fed the type-ration carrying 63.7% of unmodified sweet potato starch.

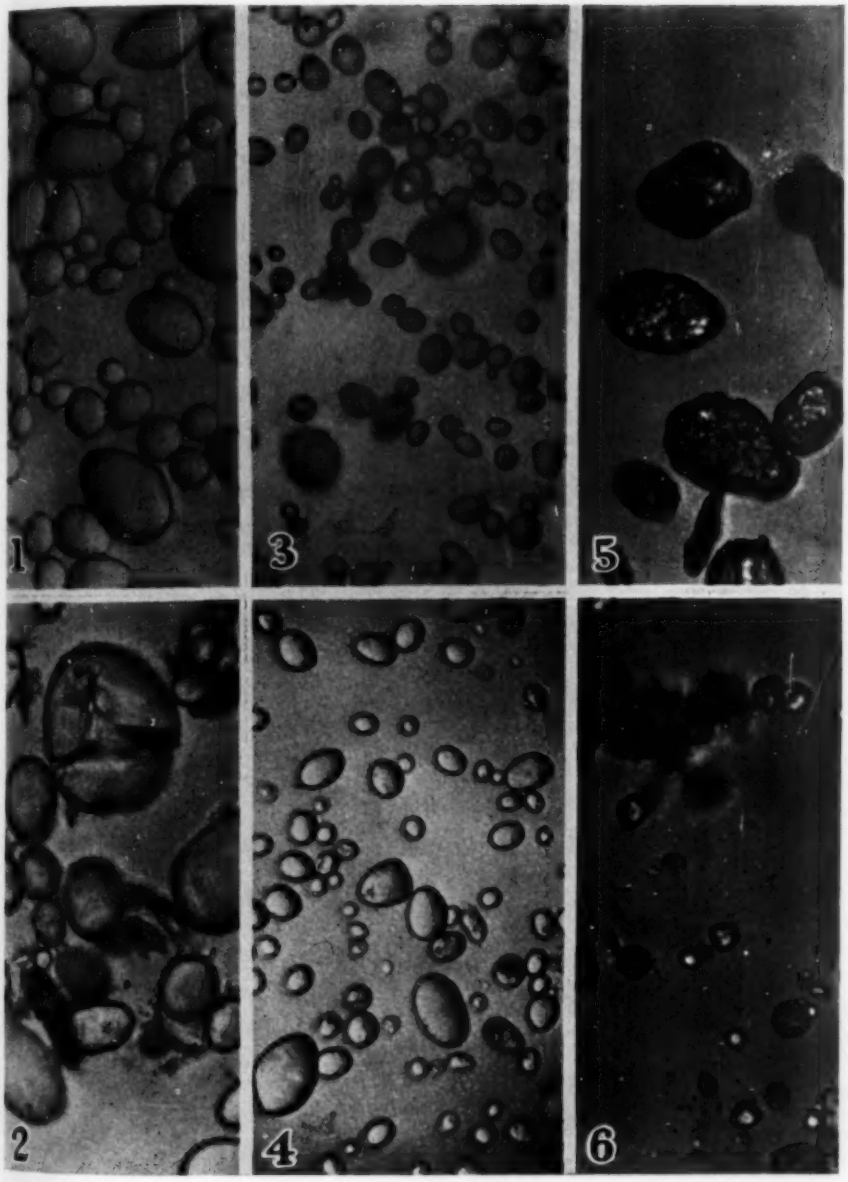
1
3
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PLATE 2

EXPLANATION OF FIGURES

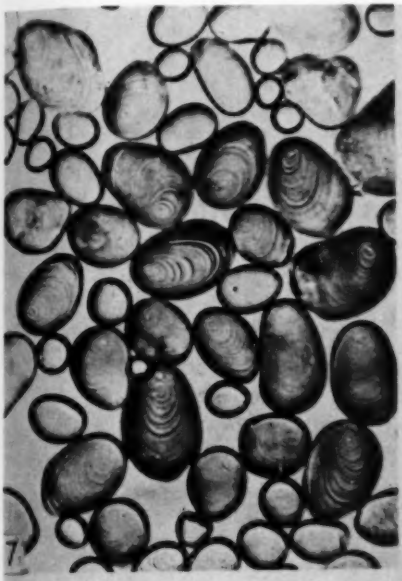
Photomicrographs (× 110) of:

7 Granules of unmodified potato starch 2.

8 Granules of potato starch 2 (in 10% slurry) after digestion with U.S.P. pancreatin at 38°C. for two hours in presence of NaCl and phosphate buffer (pH 7.0).

9 Granules of unmodified wheat starch 7 (laboratory-prepared).

10 Granules of wheat starch 7 (in 10% slurry) after digestion with U.S.P. pancreatin under same conditions as potato starch 2.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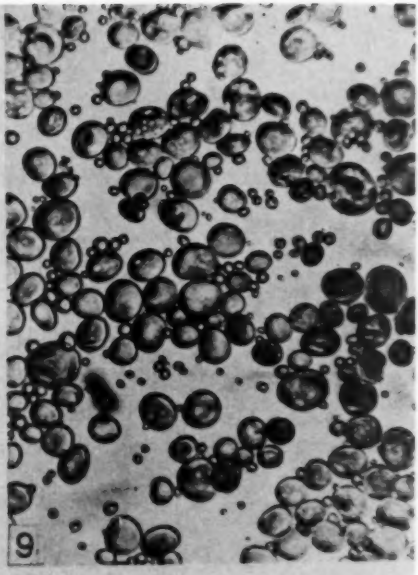
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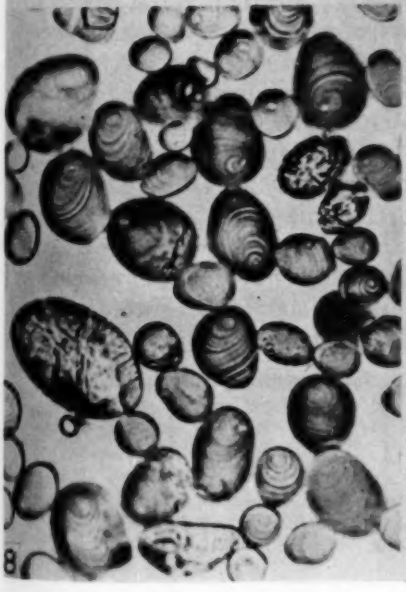
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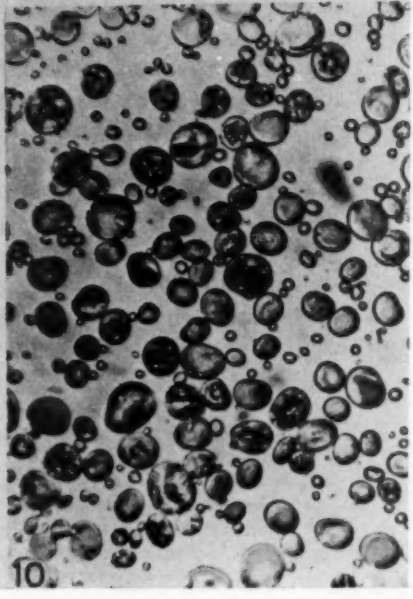
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FURTHER STUDIES ON AMINO ACID IMBALANCE PRODUCED BY GELATIN IN RATS ON NIACIN-TRYPTOPHAN LOW RATION [1]

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THREE FIGURES

(Received for publication April 9, 1951)

INTRODUCTION

The effect of tryptophan-deficient proteins (wheat gluten, zein, gelatin) or acid hydrolysates of fibrin or casein on the precipitation of an amino acid imbalance in the growing rat receiving a niacin-low diet has been well established (Krehl et al., '46a, b, c). It was noted that if dextrin, glucose, or cornstarch were substituted for the sucrose in the diet, the growth inhibition caused by the corn or wheat gluten diet was relieved, while little effect in counteracting the growth depression resulting from the casein-gelatin diet could be observed. The growth-depressing effect of glycine, DL-phenylalanine and DL-threonine (Henderson et al., '47; Hankes et al., '48), although demonstrable on the casein-sucrose diet,

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could not be induced on a diet with dextrin as the carbohydrate source. It has been suggested that the action of dextrin depends upon the establishment of a more favorable medium for bacterial synthesis of vitamins, or that because of its slower digestion, the site of absorption is altered, increasing the availability of synthesized vitamins to the host (Mannering et al., '44). Teply et al. ('47) demonstrated an increase in niacin in the cecal contents of rats receiving a dextrin diet.

Since it had been shown (Hankes et al., '50) that DL-phenylalanine and DL-threonine, fed at the approximate level at which they occur in gelatin, did not produce a significant growth depression when fed with a dextrin diet, it was suggested that possibly other factors exist in gelatin that increase the susceptibility of the rat to an amino acid imbalance. Schweigert and Pearson ('48) concluded that the effect of 12% gelatin was not an inhibition of the tryptophan-to-niacin conversion when they noted an immediate increase in the excretion of niacin and its methylated derivative (N'-methylnicotinamide) after administration of a test dose of tryptophan. Rosen and Perlsweig ('49) presented evidence which indicated that gelatin impaired the tryptophan-to-niacin metabolic pathway and that niacin could overcome the inhibition, while large doses of tryptophan (25 mg) would afford relief to the animal for only short periods of time, with subsequent relapse if no more tryptophan were made available. This was compatible with the results of previous work, since these investigators had used 100-mg doses of tryptophan.

Following the demonstration that DL-threonine, DL-phenylalanine and glycine depressed growth on a low casein-sucrose diet only when supplements of L-cystine or DL-methionine were provided (Hankes et al., '49), it seemed expedient to determine whether a similar effect could be achieved with gelatin. The work described here is a further investigation into the growth inhibition in the rat caused by gelatin, and the pos-

sible role of L-cystine and various amino acids that occur in gelatin, on the initiation of a condition of amino acid imbalance.

EXPERIMENTAL

The methods employed in the handling of the animals were similar to those previously described (Hankes et al., '49). Male weanling rats of the Sprague-Dawley strain, weighing between 40 and 50 gm, were maintained in individual cages and allowed to eat ad libitum for a 5-week experimental period. Weighings were made at weekly intervals.

The percentage composition of the basal diet was as follows: dextrin (sucrose) 81.8, corn oil 5, casein 9, salts IV (Hegsted et al., '41) 4, L-cystine 0.2. Vitamins were added so as to provide, in milligrams per 100 gm of diet: thiamine 0.2, riboflavin 0.3, pyridoxine 0.25, calcium DL-pantothenate 2, choline chloride 100, inositol 10, biotin 0.01, and folic acid 0.02. Halibut liver oil diluted with corn oil and fortified with vitamins E and K was given as a supplement once weekly by dropper to provide approximately the following daily intake: vitamin A 400 I.U., vitamin D 4 I.U., 2-methyl-1,4-naphthoquinone 0.06 mg, and alpha-tocopherol 0.7 mg. All supplements to the basal diet were at the expense of the carbohydrate.

In one of the experiments certain groups of animals were selected after the 5-week experimental period for analysis of the cecum. The contents were weighed and washed into a flask, then neutralized and diluted to appropriate volume. They were then assayed by the method of Krehl, Strong and Elvehjem ('43) for "free" niacin; i.e., with no previous acid or alkaline hydrolysis. Dry weight determinations were found to be consistent and varied only slightly from 83% moisture.

The composition of the amino acid mix simulating 6% gelatin was calculated from the values given by Block and Bolling ('48). Growth was represented as per cent of the weight gains of negative controls throughout the experiments and

this was similar to the technique employed by Henderson et al. ('47), which required a growth inhibition amounting to 60% or less of the growth of the control group receiving the basal ration with no supplementary niacin. Animals also had to exhibit the typical signs attributed to a niacin-deficient condition, which include a porphyrin exudate around the nose and eyes, unkempt hair, and general emaciated appearance. Although these symptoms are not necessarily specific for the niacin-tryptophan deficiency, they helped to differentiate between animals inhibited by an amino acid imbalance in the presence of cystine and the growth depression observed when supplements of cystine or methionine were denied. In these cases a growth inhibition, which niacin or tryptophan was ineffective in overcoming, was the only observable external effect.

RESULTS

The influence on growth of gelatin, acid-hydrolysed gelatin,[2] and certain combinations of amino acids occurring in gelatin, is presented in table 1. With either L-cystine or DL-methionine, 6% gelatin proved to be an effective growth inhibitor when the dextrin ration was used. However, in the absence of the sulfur amino acids growth increased to about four-fifths of that of the negative controls and the animals all appeared normal and healthy. A similar, although not as pronounced, remission of the inhibition was noted in the animals receiving the sucrose as the carbohydrate. The inhibition obtained with the acid-hydrolysed gelatin and the relief afforded by the omission of cystine indicated that the free amino acids of the protein were acting, in producing

[2] Acid-hydrolysed gelatin was prepared from crude gelatin by hydrolysing with 10 volumes of 3N sulfuric acid for 24 huors at 15 lb. pressure in the autoclave. After neutralizing with barium hydroxide, the barium sulfate was removed by filtration and the residue washed three times with hot water. The combined filtrate and washings were concentrated *in vacuo* to a syrup which was dried in a vacuum oven at 50°C. After grinding, the light brown powder was stored until incorporated into the diet. The substance gave a negative biuret test.

TABLE 1

Effect of gelatin, acid-hyydrolysed gelatin and certain amino acids in the presence of cystine on rat growth

	9% CASEIN BASAL RATION	SUPPLEMENT	0.2% L-CYSTINE	DEXTRIN			SUCROSE		
				No. of animals	Ave. 5-wk. gain	% gain of neg. controls	No. of animals	Ave. 5-wk. gain	% gain of neg. controls
					gm			gm	
(1)	Basal	None	+	11	122 ± 3 [1]	100	11	69 ± 4	100
(2)	Basal	6% Gelatin	+	15	50 ± 4	41	6	14 ± 3	20
(3)	Basal	6% Gelatin + 0.2% DL-methionine	—	3	40 ± 6	33			
(4)	Basal	6% Gelatin	—	10	98 ± 5	81	6	43 ± 4	62
(5)	Basal	6% Acid-hydrolysed gelatin	+	9	44 ± 4	36	3	9 ± 2	13
(6)	Basal	6% Acid-hydrolysed gelatin	—	3	87 ± 15	71			
(7)	Basal	1.5% Glycine	+	3	110 ± 6	90			
(8)	Basal	0.18% DL-threonine + 0.24% DL-phenylalanine + 1.5% glycine	+	6	87 ± 5	71	3	8 ± 4	12
(9)	Basal	Same as 8 except 0.2% DL-methionine added	—	3	84 ± 15	69	3	16 ± 3	23
(10)	Basal	0.18% DL-threonine + 0.24% DL-phenylalanine + 1.5% glycine	—	3	65 ± 9	53			
(11)	Basal	Same as 10 except 0.9% L-proline and 0.6% L-arginine added	+	6	100 ± 8	82			
(12)	Basal	Same as 11 (A.A.M.1)[2]	—	3	70 ± 7	57			
(13)	Basal	0.9% L-proline + 0.6% L-arginine	+	3	158 ± 3	129			
(14)	Basal	Same as 13	—	3	68 ± 4	56			

[1] Standard error, $\sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$

[2] A.A.M.1 — Amino acid mix 1. This mix was made up to the approximate percentage ratio of certain amino acids occurring in gelatin. To 100 gm of ration were added 3.24 gm of the mix, composed of: L-arginine hydrochloride 0.56 gm, DL-phenylalanine 0.24 gm, DL-threonine 0.18 gm, glycine 1.5 gm, and L-proline 0.92 gm.

the growth depression, in the same manner as the whole gelatin. Therefore, the effect appeared to be one of an amino acid imbalance.

Although threonine, glycine and phenylalanine, in the presence of methionine or cystine, were effective growth inhibitors on the casein-sucrose diet, little effect was obtained with these amino acids on the dextrin ration. The addition of L-proline and L-arginine, both of which are found in abundance in gelatin, to the other three amino acids was equally without effect. In fact, a slight growth stimulation was observed (group 13) when these two animo acids were fed by themselves. It was noted that in all cases where the amino acid combinations were fed in the absence of either L-cystine or DL-methionine, even though the animals failed to grow appreciably, they never exhibited the external symptoms of the niacin-tryptophan deficient condition, indicating that probably cystine or methionine was limiting growth under the conditions imposed.

At least two possible explanations for this augmentation by cystine of the growth depression and deficiency symptoms due to gelatin exist: (1) An alteration in the bacterial population of the intestine which either increased the utilization of the available tryptophan by certain bacteria, or inhibited the synthesis of niacin. (2) The cystine (acting by sparing methionine) was in some way increasing the niacin requirements of the tissues. Table 2 shows that the influence of cystine in precipitating an amino acid imbalance leading to a niacin-tryptophan deficiency was not due to inhibition of the synthetic action of the bacteria producing niacin. The "free" niacin of the cecal material was determined, since it was thought that this would be a better indication of the amount of niacin which would be available to the rat. Because the level remains relatively constant on a given diet, an equilibrium between the niacin synthesized and the niacin utilized by the organisms must have been reached and the excess should be absorbed. As can be seen, this "free" niacin remained remarkably constant throughout the various

TABLE 2

"Free" niacin content of the cecal material in rats receiving dextrin or sucrose rations

9% CASEIN BASAL RATION	SUPPLEMENT	0.2% L-CYSTINE	DEXTRIN			SUCROSE		
			Ave. 5-wk. gain	Free niacin per gm wet cecal material	Weight of wet cecal contents	Ave. 5-wk. gain	Free niacin per gm wet cecal material	Weight of wet cecal contents
			gm	*μg*	*gm*	*gm*	*μg*	*gm*
(1) Basal	None	+	123 (7) [1]	25.0 ± 2.4 [2]	1.40	64 (7)	11.8 ± 0.6	0.95
(2) Basal	6% Gelatin	+	56 (5)	27.5 ± 4.0	1.23	15 (5)	16.3 ± 3.4	0.77
(3) Basal	0.18% DL-threonine + 0.24% DL-phenylalanine + 1.5% glycine	+	88 (6)	25.2 ± 1.9	1.47	8 (2)	19.1 ± 2.0	1.10
(4) Basal	6% Acid-hydrolysed gelatin	+	28 (2)	21.5 ± 5.5	0.73	10 (2)	18.2 ± 6.0	1.00
(5) Basal	6% Acid-hydrolysed gelatin	—	86 (3)	27.2 ± 0.9	1.50			
(6) Basal	6% Gelatin	—	98 (7)	25.8 ± 3.7	2.10	44 (3)	14.1 ± 3.5	0.87
(7) Basal	6% Gelatin + 0.2% DL-methionine	—	33 (2)	24.0 ± 7.0	1.50			
(8) Basal	0.18% DL-threonine + 0.24% DL-phenylalanine + 1.5% glycine + 0.2% DL-methionine	—	56 (3)	22.9 ± 2.7	1.50			
(9) Basal	0.92% L-proline + 0.56% L-arginine	+	158 (3)	30.5 ± 3.2	2.28			

[1] Numbers in parentheses indicate number of animals.
[2] Standard error.

groups fed a given carbohydrate, despite the supplement or the presence of cystine or methionine. The level of niacin produced on the sucrose diet was somewhat lower than that produced on the dextrin ration, thus providing further evidence that the increased synthesis of niacin due to the dextrin provides the protection needed to prevent an imbalance when individual amino acids are added. To test the assumption that gelatin produced a more severe amino acid im-

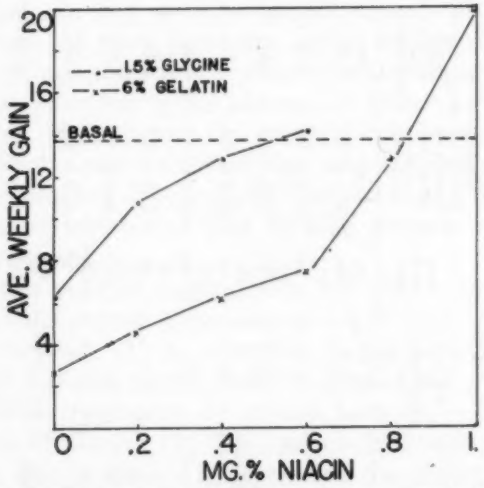

Fig. 1 The effect of increasing amounts of niacin on the gelatin and glycine inhibition in the rat. Each point represents an average of three rats.

balance, and hence required larger amounts of niacin in order to overcome the resultant condition, the experiment represented by figure 1 was performed. About 0.3 mg % more niacin was required to bring the gelatin-inhibited animals to the level of the basal controls than was needed by the glycine-inhibited animals. Since glycine does not inhibit growth on the dextrin diet, it would seem that the extra niacin made available by intestinal synthesis when animals were fed this carbohydrate was sufficient to counteract the inhibition due

to the glycine (and presumably to the other amino acid inhibitors), but was insufficient to overcome the inhibition from the gelatin.

In studying further what amino acid combinations in gelatin increase the niacin requirement, an amino acid mix made

TABLE 3

The effect of supplements of amino acids at the approximate ratio of their occurrence in gelatin on the growth of the rat

9% CASEIN BASAL RATION	SUPPLEMENT	DEXTRIN 0.2% L-CYSTINE	No. of animals	Ave. 5-wk. gain	% Gain of neg. controls
				gm	
(1) Basal	None	+	9	122 ± 5[1]	100
(2) Basal	Simulated "6% gelatin"[2]	+	6	50 ± 7	41
(3) Basal	Simulated "6% gelatin"	—	3	102 ± 4	83
(4) Basal	A.A.M. 1 + 0.6% DL-alanine[3]	+	5	52 ± 6	42
(5) Basal	A.A.M. 1 + 0.6% DL-alanine	—	6	75 ± 2	61
(6) Basal	A.A.M. 1 + A.A.M. 2[4]	+	3	63 ± 14	51
(7) Basal	A.A.M. 1 + A.A.M. 3[5]	+	3	48 ± 13	39
(8) Basal	A.A.M. 1 + A.A.M. 2 + A.A.M. 3	—	3	63 ± 11	51

[1] Standard error.

[2] See footnote 4 for composition.

[3] A.A.M. 1 — amino acid mix 1. See footnote 2, table 1.

[4] Amino acid mix 2 (A.A.M. 2): To 100 gm of ration were added 1.25 gm of amino acid mix composed of: L-histidine 0.06 gm, DL-serine 0.20 gm, DL-leucine 0.22 gm, DL-valine, 0.15 gm, and L-glutamic acid 0.62 gm.

[5] Amino acid mix 3 (A.A.M. 3): To 100 gm of ration were added 1.58 gm of amino acid mix composed of: DL-aspartic acid 0.37 gm, DL-alanine 0.6 gm, L-lysine 0.5 gm, L-tyrosine 0.012 gm, and DL-isoleucine 0.1 gm.

to simulate 6% gelatin[3] was employed. The results are shown in table 3. The simulated "6% gelatin" produced the same depression of growth in the presence of cystine as did the gelatin or the gelatin hydrolysate, and again the removal

[3] The amino acid mix made to simulate 6% gelatin had the follwoing composition in grams per 100 gm of ration: L-arginine hydrochloride 0.56; L-histidine hydrochloride 0.06, DL-phenylalanine 0.24, L-cystine 0.006, DL-methionine 0.048, DL-leucine 0.22, DL-valine 0.15, L-glutamic acid 0.62, DL-aspartic acid 0.37, DL-alanine 0.6, L-lysine 0.5, L-tyrosine 0.012, DL-threonine 0.18, DL-isoleucine 0.1, glycine 1.5, DL-serine 0.20, and L-proline 0.92.

of the cystine resulted in a significant growth response. Groschke and Briggs ('46) had found that 0.5% DL-alanine along with 2% glycine and 0.5% L-arginine were effective in producing the niacin-tryptophan deficiency syndrome in the chick. When 0.6% DL-alanine was added to amino acid mix 1, which already contained DL-threonine, DL-phenylalanine and L-proline in addition to glycine and L-arginine, a severe growth inhibition and deficiency symptoms were produced

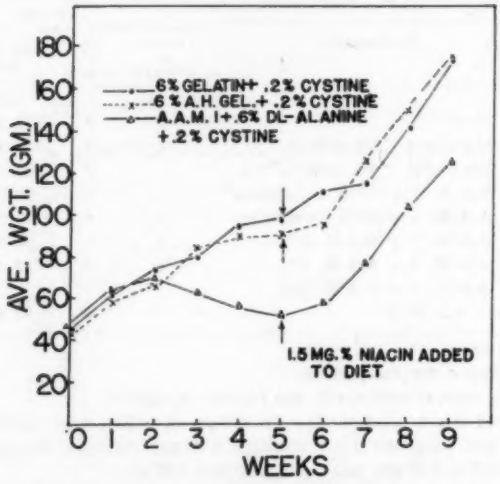

Fig. 2 Growth response to supplements of 1.5 mg % of niacin by rats whose growth had been inhibited by gelatin, acid-hydrolysed gelatin and amino acid mix 1 plus alanine.

in the presence of supplemental cystine (group 4). That these symptoms were analogous to those developed with gelatin or acid-hydrolyzed gelatin may be seen from figure 2. One and one-half milligrams per cent of niacin proved effective in relieving the growth depression to about the same extent in all cases. Omission of the cystine increased the growth somewhat, and deficiency symptoms concomitant with the niacin-tryptophan deficiency were not observed. Of interest was the growth depression produced when amino acid mixes

1 and 2 were fed with 0.2% cystine. Although this combination of amino acids did not contain DL-alanine, deficiency symptoms appeared, but the growth inhibition was not so severe. However, when amino acid mix 3 was substituted in place of amino acid mix 2, a severe deficiency developed. Since this combination contained DL-alanine, it seemed that this substance was of primary importance in the precipitation of an amino acid imbalance by gelatin, but that sheer numbers of amino acids were also effective. Animals receiving amino acid mixes 1, 2 and 3 but no cystine did not show the external deficiency symptoms, except for growth inhibition.

The series of experiments shown in table 4 were made to see whether DL-alanine could induce the niacin-tryptophan deficiency condition in the rat if all the amino acids of amino acid mix 1 were not present. The results show that DL-alanine in conjunction with cystine was without effect in producing a growth depression or deficiency symptoms. Again the limiting nature of cystine presented itself, for in every case where no cystine was added to the diet growth was limited to 12 to 15 gm per week. A comparison between group I, which received 0.2% cystine, and group II, from which it was omitted, illustrates this point. Hankes et al. ('49) had shown that on a casein-sucrose diet removal of the cystine reduced the growth of negative controls only slightly below that of animals receiving the cystine supplement, indicating that on such a ration growth was limited by the tryptophan to about the same extent as it was by the cystine. The increased growth on our dextrin diet made cystine or methionine the restricting amino acid, and supplemenation with either niacin or tryptophan had no effect. Therefore a niacin-tryptophan deficiency cannot be induced on this diet, because the deficiency of the sulfur amino acids becomes apparent before that of tryptophan. This would explain why amino acid mixes 1, 2 and 3, which would be equivalent to the simulated "6% gelatin", minus the cystine and methionine, produced only a growth inhibition (group 8, table 3).

TABLE 4

Influence of DL-*alanine and amino acids associated with gelatin in producing an amino acid imbalance in the rat*

9% CASEIN BASAL RATION	SUPPLEMENT + 0.2% L-CYSTINE	DEXTRIN		
		No. of animals	Ave. 5-wk. gain	% Gain of cystine supplemented neg. controls
			gm	
I				
(1)	None	7	126 (96–165)[1]	100
(2)	0.6% DL-alanine + 0.18% DL-threonine	3	147 (142–160)	116
(3)	0.6% DL-alanine + 0.24% DL-phenylalanine	3	119 (111–133)	95
(4)	0.6% DL-alanine + 0.24% DL-phenylalanine + 0.18% DL-threonine	3	146 (136–154)	116
(5)	0.6% DL-alanine + 1.5% glycine	3	101 (87–116)	80
(6)	0.6 DL-alanine + 1.5% glycine + 0.56% L-arginine	3	114 (109–121)	91
(7)	0.6% DL-alanine + 0.56% L-arginine + 0.92% L-proline	3	106 (81–142)	84
(8)	0.6% DL-alanine + 1.5% glycine + 0.92% L-proline	3	110 (108–112)	87
(9)	0.6% DL-alanine + 0.92% L-proline	3	118 (107–136)	94
II	Supplement — cystine omitted			
(1)	None	3	65 (58–67)	52
(2)	0.6% DL-alanine + 0.18% DL-threonine	3	77 (74–82)	61
(3)	0.6% DL-alanine + 0.24% DL-phenylalanine	3	64 (59–69)	51
(4)	0.6% DL-alanine + 0.24% DL-phenylalanine + 0.18% DL-threonine	3	64 (53–77)	51
(5)	0.6% DL-alanine + 1.5% glycine	3	72 (67–84)	57
(6)	0.6% DL-alanine + 1.5% glycine + 0.56% L-arginine	3	66 (56–72)	52
(7)	0.6% DL-alanine + 0.56% L-arginine + 0.92% L-proline	3	67 (60–75)	53
(8)	0.6% DL-alanine + 1.5% glycine + 0.92% L-proline	3	82 (79–83)	65

[1] Numbers in parentheses represent range of gains.

Figure 3 shows how increasing levels of L-cystine intensify the growth inhibition produced by gelatin. At 0.2% nearly maximum inhibition is obtained.

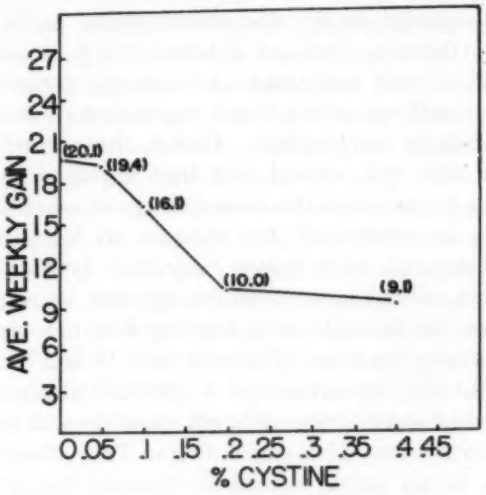

Fig. 3 The influence of L-cystine in aggravating a growth inhibition produced by gelatin. Each number in parentheses represents an average weekly gain of three or more animals over a 5-week period.

DISCUSSION

It appears that a delicate balance exists between cystine (methionine) and tryptophan as limiting amino acids in a 9% casein diet. The growth differences observed between animals receiving gelatin or the simulated "6% gelatin" (group 3, table 3) and the amino acids of gelatin without cystine or methionine (group 8, table 3) indicate that the small amounts of cystine (0.006 gm/100 gm diet) and methionine (0.048 gm/100 gm diet) contributed by the amino acid mix were sufficient to support growth only slightly inferior to that of the control animals. If the level of either of these amino acids were raised, growth inhibition due to the limited available tryptophan in the diet resulted.

On the casein-dextrin diet sufficient niacin must have been synthesized in the intestinal tract, or the dietary tryptophan was made more available by the slower digestion of the dextrin, so that the tryptophan required in the diet was reduced, while the requirement for the sulfur amino acids remained unaffected. Gelatin, then, at a level of 6%, could provide enough cystine and methionine to increase growth substantially, but growth was still not at a rapid enough rate to utilize all the available tryptophan. Under these conditions the tryptophan was not wasted and that supplied by the diet was enough to maintain the animal in good condition. Swift et al. ('34) demonstrated that rats on an 8% milk protein diet supplemented with 0.24% L-cystine synthesized more body protein, stored more food energy and excreted less nitrogen than did animals on a similar diet but receiving no cystine. Others workers (Womack and Rose, '41; Mulford and Griffith, '42) have reported a growth stimulating action of cystine, in the presence of small quantities of methionine, when fed with 9 and 18% casein diets. This stimulation was thought to be an actual metabolic increase initiated by the cystine. If this is so, an increase in the cystine or methionine content of an amino acid imbalanced diet must have accelerated the metabolism or utilization of the excessive amino acids. This situation exerted greater demands on the tryptophan, either for tissue synthesis or for conversion of the tryptophan to niacin, and the dietary source was incapable of meeting these new requirements, with the resulting development of the characteristic niacin-tryptophan deficiency syndrome. Evidently more tryptophan is needed in the metabolism of alanine in the presence of sufficient numbers of other animo acids than is required for metabolism of the amino acids themselves.

The evidence at hand neither refutes nor confirms the idea that gelatin interferes directly with the tryptophan-niacin relation, but it would seem more likely that instead of a direct impairment of the scheme, an excess of amino acids,

as provided by gelatin, merely requires more tryptophan in the presence of adequate amounts of cystine for their assimilation into body protein.

Recent work by Williams et al. ('50) suggests that the liver pyridine nucleotides in the young growing rat arise entirely from tryptophan, while niacin appears to have little effect in increasing the liver coenzyme level. If this be the case, and the tryptophan continues to synthesize the coenzymes at the same time extra demands are made by the gelatin, a deficiency condition could develop. However, it is difficult to understand why niacin is so much more effective than tryptophan in counteracting the amino acid imbalance brought on by gelatin and other tryptophan-deficient proteins, unless an adequate level of dietary niacin maintains the coenzyme level high enough so that dietary tryptophan may be utilized for structural purposes rather than coenzyme synthesis. Further work on the problem of the influence of gelatin on liver pyridine nucleotides is in progress.

SUMMARY

The influence of 6% gelatin in creating an amino acid imbalance in the rat receiving a 9% casein-dextrin diet has been reported.

If no supplemental L-cystine or DL-methionine were added to the diet of animals receiving the gelatin, only slight growth inhibition was noted. Addition of L-cystine or DL-methionine produced a severe growth inhibition, curable with 1.5 mg % of niacin.

Fed in conjunction with a given carbohydrate, L-cystine did not alter cecal niacin levels, so improved growth in the absence of L-cystine was not due to changes in the intestinal flora. Improved growth on the dextrin diet was considered to be a result of increased synthesis of niacin by the intestinal bacteria.

The combination of L-proline, DL-threonine, DL-phenylalanine, glycine, L-arginine and DL-alanine, in the presence of L-cystine, was the most effective in producing the growth

inhibition and deficiency symptoms analogous to those induced by gelatin. DL-Alanine seemed to be necessary, for in its absence no symptoms were observed.

The increased growth on the low casein-dextrin diet and the limiting nature of L-cystine and DL-methionine in the diet, which induced a cystine deficiency before the tryptophan became the limiting amino acid, were attributed to failure to produce the amino acid imbalance syndrome in the absence of supplemental sulfur amino acids.

LITERATURE CITED

BLOCK, R. J., AND D. BOLLING 1948 Amino acid composition of proteins and foods. Charles C. Thomas, Springfield, Ill., p. 300.

GROSCHKE, A. C., AND G. M. BRIGGS 1946 Inhibitory action of certain amino acids on chicks receiving nicotinic acid-low diets. J. Biol. Chem., *165:* 739.

HANKES, L. V., L. M. HENDERSON, W. L. BRICKSON AND C. A. ELVEHJEM 1948 Effect of amino acids on the growth of rats on niacin-tryptophan deficient rations. Ibid., *174:* 873.

HANKES, L. V., L. M. HENDERSON AND C. A. ELVEHJEM 1949 Effect of cystine and threonine on the growth of rats receiving tryptophan-deficient rations. Ibid., *180:* 1027.

HANKES, L. V., R. L. LYMAN AND C. A. ELVEHJEM 1950 Effect of niacin precursors on growth of rats fed tryptophan-low rations. Ibid., *187:* 547.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. Ibid., *138:* 459.

HENDERSON, L. M., T. DEODHAR, W. A. KREHL AND C. A. ELVEHJEM 1947 Factors affecting the growth of rats receiving niacin-tryptophan deficient diets. Ibid., *170:* 261.

KREHL, W. A., L. M. HENDERSON, J. DE LA HUERGA AND C. A. ELVEHJEM 1946a Relation of amino acid imbalance to niacin-tryptophan deficiency in growing rats. Ibid., *166:* 531.

KREHL, W. A., P. S. SARMA AND C. A. ELVEHJEM 1946b The effect of protein on the nicotinic acid and tryptophan requirement of the growing rat. Ibid., *162:* 403.

KREHL, W. A., P. S. SARMA, L. J. TEPLY AND C. A. ELVEHJEM 1946c Factors affecting the dietary niacin and tryptophan requirement of the growing rat. J. Nutrition, *31:* 85.

KREHL, W. A., F. M. STRONG AND C. A. ELVEHJEM 1943 Determination of nicotinic acid — modification in the microbiological method. Ind. Eng. Chem., Anal. Ed., *15:* 471.

MANNERING, G., D. ORSINI AND C. A. ELVEHJEM 1944 Effect of the composition of the diet on the riboflavin requirement of the rat. J. Nutrition, *28:* 141.

MULFORD, D. J., AND W. H. GRIFFITH 1942 Choline metabolism. VIII. The relation of cystine and of methionine to the requirement of choline in young rats. Ibid., *23:* 91.

ROSEN, F., AND W. A. PERLSWEIG 1949 The effect of gelatin on the transformation of tryptophan to niacin in rats on low casein diets. J. Biol. Chem., *177:* 163.

SCHWEIGERT, B. S., AND P. B. PEARSON 1948 Further studies on the metabolism of tryptophan and nicotinic acid by the rat. Ibid., *172:* 485.

SWIFT, R. W., O. J. KAHLENBERG, LE ROY VORIS AND E. B. FORBES 1934 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. I. The effects of cystine decieney. J. Nutrition, *8:* 197.

TEPLY, L. J., W. A. KREHL AND C. A. ELVEHJEM 1947 The intestinal synthesis of niacin and folic acid in the rat. Am. J. Physiol., *148:* 91.

WILLIAMS, J. N., JR., P. FEIGELSON AND C. A. ELVEHJEM 1950 Relation of tryptophan and niacin to pyridine nucleotides of tissue. J. Biol. Chem., *187:* 597.

WOMACK, M., AND W. C. ROSE 1941 The partial replacement of dietary methionine by cystine for purposes of growth. Ibid., *141:* 375.

NITROGEN LOSS IN THE FECES: THE VARIABILITY OF EXCRETION IN NORMAL SUBJECTS ON CONSTANT DIETARY INTAKES

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FIVE FIGURES

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In metabolic balance studies for nitrogen, lack of adequate facilities for the collection and analysis of stools has led numerous workers to omit stool analyses and to assume that the fecal nitrogen value would be 10% of the dietary intake of this element. The invalidity of this assumption, however, is evident from a number of investigations which have shown the lack of relationship between fecal nitrogen and the nitrogen in the dietary intake (Prausnitz, 1897; Schmidt and Strasburger, '03; Shorr, '43).

Reifenstein et al. ('45) recently tabulated the fecal nitrogen data of a group of patients studied by Schamberg et al. ('13) and added the analyses from 6 cases of their own. A graph of these combined data, in which nitrogen intakes ranged from 3 to 38 gm per 24 hours, showed well the lack of relation between fecal and intake nitrogen. Instead of being a constant 10% of the intake nitrogen, the fecal loss ranged from 45% of the intake on low levels of intake to only 3% on high intake levels.

Since this report appeared to suggest that fecal nitrogen varies little in absolute amount, another way of avoiding stool analyses has been suggested — the use of an arbitrary estimated figure for the daily fecal nitrogen loss, such as

1.3 gm. This figure happened to be the average daily loss of the group reviewed by Reifenstein; the range variation in this group, however, was considerable, being from 0.72 to 2.10 gm.

In view of the fact that Reifenstein's was a heterogeneous series in which variation was to be expected, we have recorded the fecal nitrogen losses in a relatively homogeneous group of 4 healthy young men. These subjects were on constant dietary intakes for 4 to 5 months at a time and, except for one series of periods in one subject, the group of 4 received exactly the same dietary intake. Under these near-optimum conditions for constancy of intake and, theoretically, of excretion, what was the variation in the fecal nitrogen excretion?

PROCEDURE

The studies which afforded this opportunity to survey variation in fecal nitrogen excretion were the immobilization experiments of Deitrick, Whedon and Shorr ('48; Whedon, Deitrick and Shorr, '49) carried out in the Metabolism Ward of the New York Hospital. They were performed in two parts. In the first, 4 normal healthy young men were studied before, during, and following a 6- to 7-week immobilization on standard hospital (fixed) beds, immobilization being standardized by plaster casts extending from waist to toes. In the second part, three of the original subjects underwent an experiment identical with the first except for the fact that during the 5-week immobilization phase in casts, they were on slowly oscillating beds instead of on standard fixed beds.

The 7 studies ranged in length from 14 to 19 metabolic periods of 7 days each. In 6 of the studies the subjects received a constant dietary intake of 90 gm of protein, 14.42 gm of nitrogen. Subject E.M. throughout his first experiment received a slightly lower intake of 85 gm of protein, 13.55 gm of nitrogen. The caloric content of the diets as well as the content of calcium, phosphorus and potassium were also kept constant throughout.

METHODS

The stools were marked by giving the subjects by mouth 0.2 gm of carmine at 9 P.M. on the last day of the period. Stool specimens were collected directly into enamel containers, which were then covered and placed in a refrigerator at 5°C. Drying of the refrigerated stools was begun within 48 hours. The dried feces for the period were pooled, weighed, thoroughly ground and mixed, and stored in capped glass bottles for subsequent analysis.

Refrigeration satisfactorily prevents decomposition of stool specimens before drying is begun. This has been shown by tests in our laboratory in which the nitrogen content of portions of stools assayed after one week of refrigeration was equivalent to 96.5% of that of the portions analyzed immediately.

For analysis a slight modification of the macro-Kjeldahl method of Folin and Wright ('19) was employed in which a 0.5-gm aliquot of the dried feces was used; this was placed directly in a 500-ml Kjeldahl flask. Digestion was carried out with 20 ml of a mixture made up of 170 ml concentrated H_2SO_4, 130 ml 85% H_3PO_4 and 100 ml 3% $CuSO_4$. The volume of 40% NaOH used in distillation was 100 ml.

RESULTS

The fecal nitrogen loss during each of the 118 metabolic balance periods is given in table 1. Statistical analysis by calculation of t values (Snedecor, '46) showed that immobilization had no influence upon fecal nitrogen excretion, and that with one exception the values throughout each of the 7 studies behaved as though belonging to single groups of 14 to 19 each. The one exception to this was that the fecal nitrogen excretion in the 6 periods during the recovery phase of subject C.O.'s second study (in 1945) was significantly different ($P = < 0.01$) from that in both the control and bed rest phase. The reason for this consistent shift in fecal nitrogen in one individual during part of one experiment was not apparent.

TABLE 1

Fecal nitrogen excretion during 7 studies of immobilization, 7-day metabolic periods

	E.M. (1944)	C.O. (1944)	A.S. (1945)	S.W. (1945)	E.M. (1945)	C.O. (1945)	S.W. (1946)
	gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*	*gm/day*
	0.96						
	0.94	0.97					
	1.01	1.01					
	0.89	1.13	2.03	1.19			
Control periods	0.88	1.20	2.03	1.34			0.94
	0.83	1.46		1.20	1.29	1.56	1.65
	0.83	1.25	1.82	1.19	0.94	1.50	1.11
	1.03	1.10	1.82	1.06	1.22	1.22	1.52
	0.76	1.31	1.72	1.12	1.02	1.39	1.19
	0.89	1.29	2.08	1.07	1.50	1.36	1.33
	1.12	1.21	1.71	1.16	1.29	1.04	1.26
Immobilization periods	0.91	1.22	1.86	1.31	1.05	1.02	1.22
	1.01	1.25	1.92	1.18	1.00	1.35	1.36
	1.10	1.18	1.75	1.22	1.16	1.50	1.34
	0.95	1.26	2.22	1.32			
			1.75	1.44			
	0.90	1.18	1.72	1.22	0.93	1.63	1.34
	0.97	1.58	1.87	1.39	1.13	1.73	1.36
	0.99	1.80	2.01	1.43	0.91	1.77	1.29
Recovery periods	1.17		1.68	1.23	1.00	1.64	1.22
			1.77	1.18	1.53	1.65	1.23
			2.04	1.15			1.24
							1.26

TABLE 2

Fecal nitrogen excretion — mean for each study, range, standard deviation, standard error, and percentage fecal N/intake N

SUBJECT	YEAR	NO. OF PERIODS	NITROGEN INTAKE	FECAL NITROGEN EXCRETION, GM/DAY				PERCENTAGE Fecal N / Intake N
				Mean	Range	Standard deviation	Standard error	
			gm/day					
E.M.	1944	19	13.55	0.95	0.76–1.17	± 0.104	± 0.024	7.02
E.M.	1945	14	14.42	1.14	0.91–1.53	± 0.202	± 0.054	7.89
C.O.	1944	17	14.42	1.26	0.97–1.80	± 0.201	± 0.049	8.72
C.O.	1945	14	14.42	1.45	1.02–1.77	± 0.238	± 0.064	10.02
S.W.	1945	19	14.42	1.23	1.06–1.44	± 0.111	± 0.026	8.52
S.W.	1946	17	14.42	1.29	0.94–1.65	± 0.152	± 0.037	8.93
A.S.	1945	18	14.42	1.88	1.68–2.22	± 0.157	± 0.037	13.00

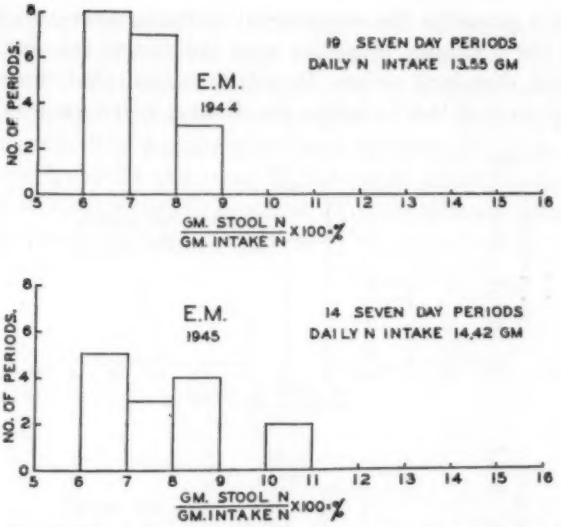
NO. OF PERIODS.
E.M.
1944
19 SEVEN DAY PERIODS
DAILY N INTAKE 13.55 GM
GM. STOOL N / GM. INTAKE N X 100 = %
E.M.
1945
14 SEVEN DAY PERIODS
DAILY N INTAKE 14.42 GM
GM. STOOL N / GM. INTAKE N X 100 = %

Fig. 1 Distribution graphs of the percentage of fecal N/intake N in the two immobilization studies on subject E.M.

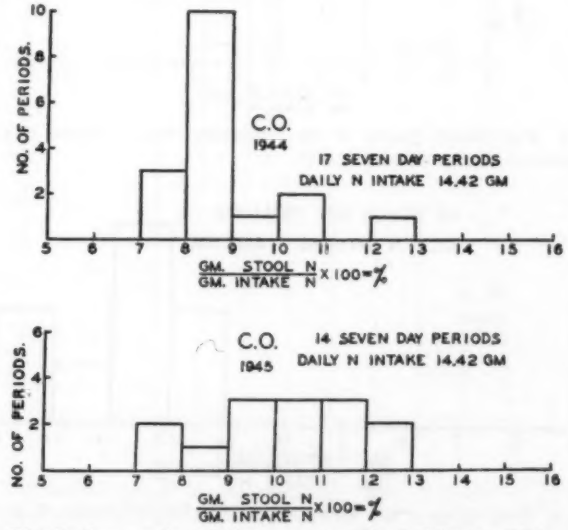
NO. OF PERIODS.
C.O.
1944
17 SEVEN DAY PERIODS
DAILY N INTAKE 14.42 GM
GM. STOOL N / GM. INTAKE N X 100 = %
C.O.
1945
14 SEVEN DAY PERIODS
DAILY N INTAKE 14.42 GM
GM. STOOL N / GM. INTAKE N X 100 = %

Fig. 2 Distribution graphs of the percentage fecal N/intake N in the two immobilization studies on subject C.O.

Table 2 presents the mean fecal nitrogen excretion during each of the 7 studies, together with the range, standard deviation and standard error. It will be noted that despite the fact that in 6 of the 7 studies the dietary nitrogen intake was

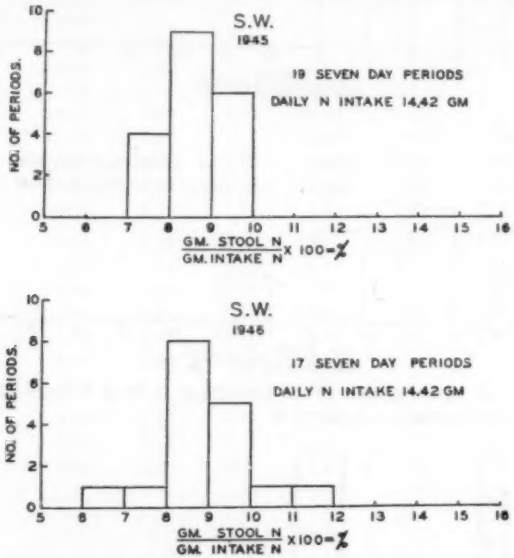
S.W.
1945
19 SEVEN DAY PERIODS
DAILY N INTAKE 14.42 GM
NO. OF PERIODS.
GM. STOOL N / GM. INTAKE N X 100 = %
S.W.
1946
17 SEVEN DAY PERIODS
DAILY N INTAKE 14.42 GM
NO. OF PERIODS.
GM. STOOL N / GM. INTAKE N X 100 = %

Fig. 3 Distribution graphs of the percentage fecal N/intake N in the two immobilization studies on subject S.W.

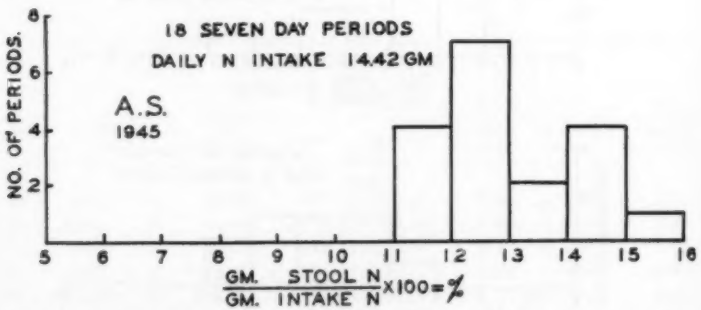

Fig. 4 Distribution graph of the percentage fecal N/intake N in the immobilization study on subject A.S.

identical, the mean fecal N in per cent of intake N varied from 7% to 13% from one study to another.

Figures 1 through 4 illustrate the variations in excretion in each study by distribution graphs of the fecal N/intake N percentages. The graphs also show the alterations in pattern from one year to the next in the same individuals; in the case of two subjects (C.O. and S. W.) the dietary intake level was the same during both years.

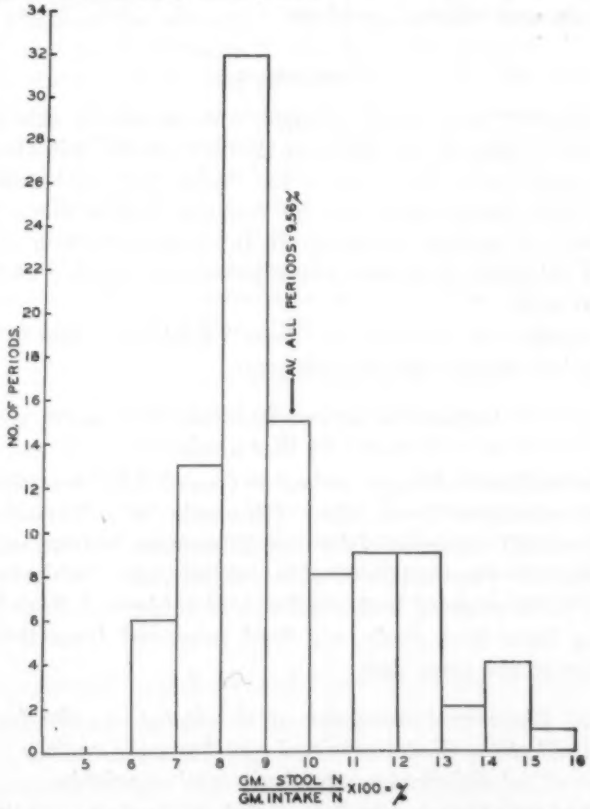

Fig. 5 Distribution graph of the percentage fecal N/intake N in the 99 metabolic periods during which the nitrogen intake was 14.42 gm daily (6 studies on 4 subjects).

Figure 5 is a distribution graph of the fecal N in per cent of intake N for the 99 seven-day metabolic periods during which the subjects received 14.42 gm of nitrogen daily. While the over-all average fecal N/intake N was not far from 10% at this intermediate dietary nitrogen level, the graph actually shows skewness, with a high peak between 8% and 9% and a tail reaching to 16%. Despite the fact that in all periods the dietary nitrogen intake was identical, the wide range of fecal nitrogen values is evident.

DISCUSSION

The variation in fecal nitrogen loss shown by this group of healthy subjects on the same dietary intake indicates that assumption of a fixed figure for daily fecal nitrogen in a given experiment would not be reliable. While there was a tendency to greater constancy in the fecal excretion of individual subjects, there was nevertheless appreciable variation here as well.

A number of reasons for these variations suggest themselves, but none is entirely adequate.

1. Greater variations in intake than were detected by diet analyses

The analyzed nitrogen values averaged 3.7% less than the values calculated from tables (Toscani, '48). Variations in intake would not account for the differences in fecal nitrogen between the two members of a subject-pair. Subjects E.M. and C.O. throughout both studies, and subjects A.S. and S.W. during their first study, ate food prepared from the same sources on the same day.

2. The mixed character of the diets; i.e., the fact that the diets contained a rather wide variety of different meats, fruits and vegetables

Mixed diets may contain somewhat varying quantities of poorly digestible nitrogenous material in addition to fully utilizable proteins. While this may in part explain the vari-

ation from period to period in individual subjects, variation in fecal nitrogen is not limited to subjects on mixed diets. McClellan, Rupp and Toscani ('30) studied the nitrogen, calcium and phosphorus metabolism of two Arctic explorers on all-meat diets. Subject K. Andersen during periods II to XII (110 days) was on a fairly constant dietary intake of 18 to 22 gm of nitrogen daily. The fecal nitrogen loss during periods VI through XII varied only moderately (3.8% to 5.1% of the intake nitrogen), but in periods II through V the percentage fecal N/intake N ranged from 2.4 to 7.1%. Subject V. Stefansson during periods II, III, IV, VII and VIII (40 days), while on an all-meat diet, had a considerably greater fecal loss in absolute amount, and his fecal N/intake N ranged from 6.5 to 10.4%. These variations in fecal nitrogen excretion on diets in which the protein was almost entirely utilizable suggest that variation may also occur in that part of the fecal nitrogen emanating from metabolic products — intestinal secretions, cast-off cells, bacteria, and so forth (Mendel and Fine, '12).

3. Errors in partitioning the stool

While there is occasionally some difficulty in separating the carmine-stained portion of the stool of one period from the unstained portion of the stool of the following period when the stool is particularly soft, this source of error is kept to a minimum if periods are of sufficient length; 6 to 7 days or more. Any mistakes in partitioning stools in shorter (three-day) periods might result in errors of considerable magnitude.

4. Alteration in bowel habit and frequency of stools

If significant changes in the frequency of stools had occurred, variation in nitrogen loss would be expected to result. The increase in stool nitrogen in chronic diarrhea (Bassett et al., '39; Harrison et al., '47) is well known and also occurs in short periods characterized by loose stools (Bassett et al., '39). Significant changes in stool frequency did not occur, however,

in the normal young subjects in these studies except for an occasional day without a bowel movement for subjects E.M. and C.O. during the immobilization phase of their first experiment.

This survey indicated that appreciable variation in fecal nitrogen excretion occurred even under optimum conditions for constancy — subjects unaffected by disease, constant dietary intakes, regularity in bowel movements, and metabolic periods of adequate length. While the variation was not extreme, it was nevertheless wide enough to indicate the necessity for direct analysis of the stools in individual experiments. Even in an experiment in which large changes in urinary nitrogen were to be expected, so that the fecal nitrogen would tend to become relatively less important, lack of specific information on the varying amounts of fecal nitrogen would cast some doubt on the validity of the conclusion reached.

The possibility of a characteristic degree of nitrogen absorption in the intestine for each individual is suggested by the fact that variation in fecal nitrogen in these studies was less marked from one period to another in the same individual than among the several individuals on the same dietary intake. The finding of differences in the fecal N expressed as percentage of intake N from one year to the next in the same subject on the same dietary intake, however, is added evidence for the view that a variety of factors influence the amount of nitrogen excreted in the feces.

SUMMARY AND CONCLUSIONS

A survey was made of the variation in fecal nitrogen excretion of 4 normal healthy subjects on constant dietary intakes for 4 to 5 months.

1. During 99 metabolic periods of 7 days each in which the nitrogen intake was identical among the 4 subjects (14.42 gm daily), the fecal nitrogen ranged from 0.91 to 2.22 gm and the mean percentage fecal N/intake N ranged from 7.9 to 13.0%.

2. Variation in fecal nitrogen excretion was more marked from one subject to another than from period to period in the same individual.

3. Since appreciable variation in fecal nitrogen loss occurred under optimum conditions for constancy, it would be unwise in an individual experiment to assume fecal nitrogen to be either 10% of the intake nitrogen or a fixed value of 1.3 gm. For reliability, direct analysis of fecal nitrogen is indispensable.

LITERATURE CITED

BASSETT, S. H., E. H. KEUTMANN, H. VAN Z. HYDE, H. E. VAN ALSTINE AND E. RUSS 1939 Metabolism in idiopathic steatorrhea. I. The influence of dietary and other factors on lipid and mineral balance. J. Clin. Invest., *18:* 101.

DEITRICK, J. E., G. D. WHEDON AND E. SHORR 1948 The effects of immobilization upon various metabolic and physiologic functions of normal men. Am. J. Med., *4:* 3.

FOLIN, O., AND L. E. WRIGHT 1919 A simplified macro-Kjeldahl method for urine. J. Biol. Chem., *38:* 461.

HARRISON, H. E., H. C. HARRISON, R. R. TOMPSETT AND D. P BARR 1947 Potassium deficiency in a case of lymphosarcoma with the sprue syndrome. Am. J. Med., *2:* 131.

MCCLELLAN, W. S., V. R. RUPP AND V. TOSCANI 1930 Prolonged meat diets with a study of the metabolism of nitrogen, calcium and phosphorus. J. Biol. Chem., *87:* 669.

MENDEL, L. B., AND M. S. FINE 1912 On the origin of fecal nitrogen. Ibid., *11:* 10.

PRAUSNITZ, W. 1897 Die chemische zuzammensetzung des kothes bei verschiedenartiger ernahrung. Zschr. Biol., *35:* 335.

REIFENSTEIN, E. C., JR., F. ALBRIGHT AND S. L. WELLS 1945 The accumulation, interpretation, and presentation of data pertaining to metabolic balances, notably those of calcium, phosphorus and nitrogen. J. Clin. Endocrinology, *5:* 367.

SCHAMBERG, J. F., T. A. KOLMER, A. I. RINGER AND G. U. RAIZISS 1913 Research studies in psoriasis. J. Cutan. Dis., *31:* 802.

SCHMIDT, A., AND J. STRASBURGER 1903 Die Faeces des Menschen im Normalen und Krankhaften Zustande. August Hirschwald, Berlin, p. 119.

SHORR, E. 1943 Deviation of fecal nitrogen excretion from values calculated as 10 per cent of nitrogen intake. Macy Conference on Metabolic Aspects of Convalescence including Bone and Wound Healing, Third Meeting, p. 68.

SNEDECOR, G. W. 1946 Statistical Methods. Iowa State College Press, Ames.

TOSCANI, V. 1948 Comparison of analyzed with calculated diets. Food Res., *13:* 187.

WHEDON, G. D., J. E. DEITRICK AND E. SHORR 1949 Modification of the effects of immobilization upon metabolic and physiologic functions of normal men by the use of an oscillating bed. Am. J. Med., *6:* 684.

UTILIZATION BY THE CHICK OF VITAMIN A FROM DIFFERENT SOURCES

I. CRYSTALLINE CAROTENE, CRYSTALLINE VITAMIN A ACETATE, AND "BLACK COD" LIVER OIL [1]

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INTRODUCTION

Since the utilization of vitamin A in its different forms shows variation between and within species, it is of importance to investigate the comparative efficiency affecting growth and liver storage of the vitamin when different sources of vitamin A used in poultry rations are presented to a single breed of chicken.

Record, Bethke and Wilder ('37) observed that the growth response of chicks to carotene or vitamin A was approximately the same when equivalent amounts were fed at low intake levels. Guilbert, Howell and Hart ('40) found, in studying different animal species, that the ratio of the relative efficiency of vitamin A to carotene widens with increasing intake levels.

Bolin, Lampman and Berg ('43) observed in young chicks that carotene contributed to liver storage of vitamin A as early as the first week. Johnson, Carrick and Hauge ('48) indicated that a high level of vitamin A storage in the liver is not essential for satisfactory growth in young chicks.

[1] Authorized for publication on October 16, 1950 as paper 1628 of the journal series of the Pennsylvania Agricultural Experiment Station. The data in this paper were presented at the 38th annual meeting of the Poultry Science Association at Guelph, Ontario, August, 1949.

This series of experiments had as its object a comparison of the efficiency of utilization (measured by rate of growth and by liver storage of vitamin A) of crystalline carotene, crystalline vitamin A acetate, and "Black Cod" liver oil as sources of vitamin A for chicks.

Throughout this paper references to vitamin A apply to vitamin A activity, regardless of whether the source was vitamin A from fish oil, carotene or some other vitamin A-active substance.

EXPERIMENTAL

Day-old Barred Plymouth Rock chicks, obtained from the breeding flock maintained by the Department of Poultry Husbandry, were used in both experiments. The chicks were kept through the 4th week of age in electrically heated starter batteries with hardware cloth floors. At the beginning of the 5th week they were transferred to unheated grower batteries located in an adjoining room where the temperature was thermostatically controlled. Water and feed were provided ad libitum. During a two-week depletion period, the chicks were fed the vitamin A-low basal diet. On the 14th day the chicks were individually weighed, wing-banded and distributed into groups as evenly as possible on a weight basis. Vitamin A supplements were added to the basal diet and fed to the chicks from the 14th day through the 8th week of age.

Individual weekly weights were recorded. The average weight of the chicks in each group was obtained by adding the average of the males to the average of the females and dividing the sum by two. The analysis of variance was made on the gains in body weight from the 14th day to the 8th week of age.

The vitamin A-low basal diet had the following percentage composition: ground wheat 35.75, ground oats 10, wheat bran 10, wheat standard middlings 10, soybean oil meal (expeller-processed) 10, dried whey 6, commercial casein 6, brewers' dried yeast 6, pulverized limestone 2.5, steamed bone meal 1.3,

iodized salt 0.3, manganese sulfate 0.05, D-activated animal sterol 0.1, and cottonseed oil 2.

The partial composition of the basal diet, determined by chemical analysis, was as follows: protein (N × 6.25) 20.94%, moisture 8.98%, ash 6.25%, ether extract 4.10%, crude fiber 4.15%, calcium 1.58%, and phosphorus 0.68%.

The supplements,[2] dissolved or suspended in cottonseed oil, were handmixed into the basal diet at frequent intervals in order to avoid appreciable loss of potency before being fed. Carotene [3] (90% beta- and 10% alpha-carotene taken as equivalent to 95% beta-carotene) was converted to International Units of vitamin A by dividing the micrograms of beta-carotene by 0.6. The vitamin A potencies of the "Black Cod" liver oil and crystalline vitamin A acetate in cottonseed oil were determined colorimetrically by the Carr-Price method referred to below.

Vitamin A was determined in pooled blood and pooled liver samples at hatching, at the end of the 14-day depletion period, and at the end of the 5th and 8th weeks. These composite samples were composed of blood or livers from 6 to 10 chicks, equally divided as to sex where possible. Vitamin A in blood plasma was determined by the method described by Kimble ('39). Vitamin A in the livers was determined by the Carr-Price colorimetric method described by the Association of Vitamin Chemists ('47). The Evelyn photoelectric colorimeter was used throughout to measure transmission of light.

RESULTS AND DISCUSSION

In experiment 1, at the end of the two-week depletion period 480 chicks were distributed into 16 groups of 30

[2] The supplements were obtained from the following sources: crystalline carotene (90% beta-, 10% alpha-) in 1-gm ampules from General Biochemicals, Inc.; "Black Cod" liver oil, lot B-20015 with a vitamin A potency of about 30,400 I.U. per gram, from the Gelatin Products Division of the R. P. Scherer Corp.; crystalline vitamin A acetate in 1-gm ampules from Distillation Products, Inc. Cottonseed oil was supplied by the Buckeye Cotton Oil Co.

[3] Throughout the paper, this mixture of beta- and alpha-carotene will be referred to as crystalline carotene.

chicks each. The basal diet was supplemented with vitamin A from three different sources; namely, crystalline carotene, crystalline vitamin A acetate and "Black Cod" liver oil. For each of these sources intake levels of 500, 1,000, 2,000, 4,000 and 8,000 I.U. per pound of diet were used.[4] A control group which received the unsupplemented basal diet was also included.

The average total weight gains for source and level of vitamin A are presented in table 1. Chicks that received 500 I.U. gained at the lowest rate in each treatment. Since the analysis of variance (table 2) indicated that there was no significant difference between treatments, it is apparent that under the conditions of this experiment normal growth, as exhibited by gain in body weight, must be supported by some intake level between 500 and 1,000 I.U. Sex, as is known, was a factor influencing growth response. Males were, on the average, heavier than females.

The interaction of treatment with intake level was highly significant (table 2), indicating that, in their effect on rate of growth, all treatments do not give the same results at the same intake level. Theoretically, as regards vitamin A intake, an optimum range may be anticipated for each source of vitamin A in its utilization by a definite species. Actually, in view of the fairly wide experimental intake levels selected, these optimum ranges tended to approximate each other and, as is seen from the total weight gains (table 1), the optimum value lay between 1,000 and 4,000 I.U. for the three sources of vitamin A used in this experiment.

The control group which received no supplement to the basal diet declined rapidly in weight and exhibited characteristic symptoms of ataxia, drowsiness, poor feather condition and optical encrustation. There were no survivors to the 6th week.

From an examination of the data in table 1 it is apparent that some minimum level of vitamin A in the blood must be

[4] International Units, when used in connection with intake levels, will refer to International Units of vitamin A per pound of diet and will be designated as I.U.

TABLE 1

Experiment 1

Gain in body weight from the 14th day to the 8th week of age and vitamin A concentration in blood plasma and liver [1]

VITAMIN A PER POUND OF DIET	TOTAL WEIGHT GAIN			VITAMIN A IN BLOOD PLASMA		VITAMIN A IN LIVER	
	Male	Female	Average	5th wk.	8th wk.	5th wk.	8th wk.
I.U.	*gm*	*gm*	*gm*	*I.U./100 ml plasma*		*I.U./gm liver*	
			Crystalline carotene				
500	419	419	419	35	36	[2]	[2]
1,000	632	546	589	83	83	[2]	2
2,000	617	531	574	123	142	4	11
4,000	694	615	655	153	189	15	55
8,000	566	525	545	188	249	26	80
			Crystalline vitamin A acetate				
500	560	479	519	54	60	2	2
1,000	618	520	569	85	100	3	6
2,000	648	583	616	170	215	14	30
4,000	609	547	578	245	278	45	116
8,000	659	552	606	254	293	173	413
			"Black Cod" liver oil				
500	554	539	546	30	63	1	2
1,000	632	566	599	63	99	3	3
2,000	585	527	556	143	188	8	13
4,000	645	524	585	209	242	42	69
8,000	585	510	548	275	313	136	274

[1] Concentration at hatching: 590 I.U. per 100 ml of plasma,
65 I.U. per gram of liver.
Concentration at end of 14-day depletion period:
48 I.U. per 100 ml of plasma,
3 I.U. per gram of liver.

[2] Not measurable.

TABLE 2

Experiment 1

Analysis of variance of total weight gains

SOURCE OF VARIATION	DEGREES FREEDOM	MEAN SQUARE	F
Treatments	2	998.42	0.16
Levels	4	40,201.01	6.48 [1]
Sex	1	235,541.20	37.96 [1]
Treatment × level	8	36,369.11	5.86 [1]
Experimental error	120	6,205.16	

[1] Significant at 1% level.

maintained before measurable liver storage of the vitamin occurs. At low intake levels most of the vitamin is conceivably employed in biological functions. At higher intake levels, significant liver storage takes place.

The amount of vitamin A in the blood plasma was measured for the purpose of determining a possible relationship between the quantity of vitamin A present in the blood and that stored in the liver . Since it was not possible to sacrifice all groups simultaneously (about three hours elapsed between the time the first and the last chicks were killed), it is felt that the values obtained are by no means absolute, because they are perhaps influenced to a slight degree by the particular digestive state prevailing in the experimental group at the time the sample was taken.

The values obtained do not indicate any simple relationship between blood plasma levels of vitamin A and liver storage of the vitamin. From table 1 it would appear that the critical plasma level for vitamin A was about 50 I.U. per 100 ml. Below this value, the chicks had apparently drawn on their final liver reserves in order to maintain the vitamin A level in the plasma.

Approximately 100 I.U. of vitamin A per 100 ml of plasma apparently comprised a threshold value which permitted accumulation of vitamin A in the liver following depletion. Above the 1,000 I.U. intake level, from the 5th to the 8th week, the increase of vitamin A stored in the liver was proportionately greater than the increase of vitamin A contained in the plasma. This effect seemed to become evident only after the level of vitamin A in the plasma had reached a value of more than 100 I.U. per 100 ml. This suggests a tendency on the part of the blood to maintain a relatively stable level of vitamin A at the expense of liver storage. However, this is not to be construed as an indication of an unvarying relationship between blood plasma vitamin A level and the amount of vitamin A which may be found in the liver, since other factors, such as previous storage and source of vitamin A, are operative.

Plasma levels of vitamin A increased with age and with increased intakes of vitamin A. There was no indication of plasma or liver saturation of vitamin A at the intake levels employed or at the ages studied.

Normal growth was not dependent upon measurable storage of vitamin A in the liver provided a sufficient quantity of the vitamin was available in the daily diet. This is evidenced by the fact that no significant difference was found in total weight gain between levels above 500 I.U. for all treatments, although these same levels differed greatly in amounts of liver-stored vitamin A.

Apparently the storage of vitamin A in the liver was a function of the quantity of vitamin A supplied by the diet. At intake levels above 1,000 I.U., crystalline vitamin A acetate was most efficient and crystalline carotene least efficient in promoting liver storage of vitamin A. Gurcay, Boucher and Callenbach ('50), using turkey poults in similar experiments, also observed, when vitamin A sufficient to support growth was contained in the diet, that crystalline vitamin A acetate was more efficient than "Black Cod" liver oil or crystalline carotene in promoting liver storage of vitamin A.

To investigate further the response of Barred Plymouth Rock chicks to vitamin A from different sources, experiment 2 was planned as an extension of experiment 1. It was desired to study the effects of feeding crystalline carotene, crystalline vitamin A acetate and "Black Cod" liver oil at high intake values. In this experiment 480 chicks at the end of a two-week depletion period were distributed into 16 groups of 30 chicks each. The basal diet was supplemented with vitamin A from the same three sources. For each source, intake levels of 6,000, 12,000, 18,000 and 24,000 I.U. were selected, plus one of 500 I.U. (identical to that used in experiment 1) as a contrasting low level. A control group was also included which received the unsupplemented basal diet.

Examination of the average total weight gains (table 3) indicates that the 500 I.U. intake level for each treatment was least effective in supporting growth. This is in agreement

with experiment 1. However, increasing the vitamin A intake, presumably beyond some optimum value, apparently resulted in a growth-retarding effect, since the average total weight gains were smaller at the highest intake levels.

No significant difference in the effects due to treatments was found (table 4), but that some operating factor exerted an influence on the results may be inferred from the value of

TABLE 3

Experiment 2

Gain in body weight from the 14th day to the 8th week of age and vitamin A concentration in blood plasma and liver[1]

VITAMIN A PER POUND OF DIET	TOTAL WEIGHT GAIN			VITAMIN A IN BLOOD PLASMA		VITAMIN A IN LIVER	
	Male	Female	Average	5th wk.	8th wk.	5th wk.	8th wk.
I.U.	*gm*	*gm*	*gm*	*I.U./100 ml plasma*		*I.U./gm liver*	
			Crystalline carotene				
500	626	530	578	56	61	1	2
6,000	679	601	640	169	234	44	74
12,000	682	592	637	219	253	126	243
18,000	681	547	614	231	258	212	377
24,000	615	581	598	270	271	303	446
			Crystalline vitamin A acetate				
500	605	478	542	54	54	2	2
6,000	660	635	648	238	255	183	316
12,000	657	623	640	263	283	482	728
18,000	620	609	614	278	288	685	1,163
24,000	679	569	624	450	356	1,139	2,291
			"Black Cod" liver oil				
500	597	520	558	52	48	2	3
6,000	654	557	606	224	220	119	219
12,000	702	528	615	255	255	425	685
18,000	639	552	596	293	258	636	1,065
24,000	629	547	588	355	355	795	1,645

[1] Concentration at hatching: 1,038 I.U. per 100 ml of plasma,
73 I.U. per gram of liver.

Concentration at end of 14-day depletion period:
105 I.U. per 100 ml of plasma,
9 I.U. per gram of liver.

"F," which approaches significance at the 5% level. A possible explanation may be found in the data presented in table 3, since the average total weight gains of all but one of the "Black Cod" liver oil groups were lower than the weights of corresponding groups in other treatments. The control group, which received no supplement to the basal diet, had no survivors to the 7th week. Sex was again found to be highly significant in growth response. Males were, on the average, heavier than females.

TABLE 4

Experiment 2

Analysis of variance of total weight gains

SOURCE OF VARIATION	DEGREES FREEDOM	MEAN SQUARE	F
Treatments	2	15,580.40	3.01
Levels	4	41,434.70	7.99 [1]
Sex	1	212,064.00	40.90 [1]
Treatment × level	8	6,495.48	1.25
Experimental error	118	5,184.76	

[1] Significant at 1% level.

Storage of vitamin A in the liver, as indicated in table 3, again appeared to be dependent on the quantity and source of the vitamin contained in the diet. At levels of intake above 500 I.U., crystalline vitamin A acetate was most efficient in promoting liver storage of vitamin A, while crystalline carotene was least efficient. Increased vitamin A intake resulted in increased concentrations of vitamin A in the blood plasma as well as in the liver. Saturation of the liver with vitamin A was not achieved under the conditions of this experiment, since each intake level up to the maximum of 24,000 I.U. showed increased liver storage of vitamin A over that on the next lower intake level. This is in agreement with results reported by Deuel et al. ('43), who supplemented the diet of laying hens with a high potency shark liver oil and obtained evidence that maximum amounts of vitamin A in the liver were found when 60,000 I.U. of vitamin A per

pound of diet were fed. No increases were noted when the diet was further supplemented.

SUMMARY

The comparative utilization by Barred Plymouth Rock chicks of vitamin A from crystalline carotene, crystalline vitamin A acetate and "Black Cod" liver oil was studied in two experiments carrying the birds to 8 weeks of age. Efficiency of utilization was judged by gain in body weight and by liver storage of vitamin A. The concentration of vitamin A in blood plasma was also studied with a view to determining possible relationships which may exist between blood content and liver stores of vitamin A. The results of these studies permit the following conclusions:

1. When sufficient vitamin A was ingested to raise the concentration in the blood to a certain level, crystalline carotene, crystalline vitamin A acetate and "Black Cod" liver oil were equally effective in sustaining a satisfactory rate of growth.

2. For the sources employed, the minimum amount of vitamin A required for satisfactory growth of the chick to 8 weeks of age was between 500 and 1,000 I.U. of vitamin A per pound of diet.

3. Excessively high vitamin A intake exerted a growth-depressing effect.

4. The amount of vitamin A present in the blood was related to the vitamin A intake.

5. A value of approximately 50 I.U. of vitamin A per 100 ml of blood plasma was observed to be critical for liver storage of the vitamin, in that the last measurable reserves of vitamin A in the liver were drawn upon to maintain the plasma value at this level.

6. A value of approximately 100 I.U. of vitamin A per 100 ml of blood plasma was apparently necessary to permit storage of vitamin A in the liver when supplementation followed vitamin A depletion.

7. The blood plasma level of vitamin A tended to remain relatively static at the expense of liver storage of the vitamin.

8. A high storage of vitamin A in the liver was not essential for satisfactory growth, provided sufficient vitamin A was supplied in the daily diet.

9. Of the sources employed, crystalline vitamin A acetate was most effective and crystalline carotene least effective in promoting concentration of the vitamin in the blood plasma and in the liver.

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LITERATURE CITED

ASSOCIATION OF VITAMIN CHEMISTS, INC. 1947 Methods of Vitamin Assay. Interscience Publishers, Inc., New York, N. Y., pp. 19–68.

BOLIN, D. W., C. E. LAMPMAN AND L. R. BERG 1943 The influence of carotene intake as supplied by dehydrated alfalfa on the storage of vitamin A and pigments in the livers of the young chick. Poultry Sci., *22:* 348–353.

DEUEL, H. J., JR., M. C. HRUBETZ, F. H. MATTSON, M. G. MOREHOUSE AND A. RICHARDSON 1943 Studies on carotenoid metabolism. IV. The effect of vitamin A intake on the carotenoid and vitamin A content of the eggs, liver, blood and body fat of hens. J. Nutrition, *26:* 673–685.

GUILBERT, H. R., C. E. HOWELL AND G. H. HART 1940 Minimum vitamin A and carotene requirements of mammalian species. Ibid., *19:* 91–103.

GURCAY, R., R. V. BOUCHER AND E. W. CALLENBACH 1950 Utilization of vitamin A by turkey poults. I. Crystalline carotene, crystalline vitamin A acetate and "Black Cod" liver oil. Ibid., *41:* 565–582.

JOHNSON, E. L., C. W. CARRICK AND S. M. HAUGE 1948 The vitamin A requirement of young chickens. Poultry Sci., *27:* 308–314.

KIMBLE, M. S. 1939 The photocolorimetric determination of vitamin A and carotene in human plasma. J. Lab. Clin. Med. *24:* 1055–1065.

RECORD, P. R., R. M. BETHKE AND O. H. M. WILDER 1937 The vitamin A requirements of chicks with observations on the comparative efficiency of carotene and vitamin A. Poultry Sci., *16:* 25–33.

EFFECTS OF CERTAIN ANTIBIOTICS ON THE GROWTH OF RATS FED DIETS LIMITING IN THIAMINE, RIBOFLAVIN, OR PANTOTHENIC ACID [1]

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In a recent study on the comparative activities of the three forms of vitamin B_6 (Linkswiler et al., '51), aureomycin was observed to increase the growth of rats fed only limiting amounts of this vitamin. The present paper deals with the effect of several antibiotics on the growth responses of rats receiving limiting amounts of thiamine, riboflavin, or pantothenic acid.

EXPERIMENTAL

The general experimental plan was as follows: groups of 5 weanling male rats were given appropriate diets deficient in one of these vitamins and, as in a bioassay, graded doses of the vitamin were then fed. In addition some of the groups also received aureomycin, streptomycin, penicillin, terramycin, chloromycetin, 3-nitro-4-hydroxyphenyl arsonic acid or sulfasuxidine incorporated into the basal ration. The levels of the vitamins are indicated in tables 1 to 4; the antibiotics

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were usually fed at the level of 50 mg per kilogram of ration; sulfasuxidine was fed at 1% of the diet.

The assay diets for riboflavin and thiamine had the following percentage composition with the appropriate vitamins omitted: sucrose 73, vitamin-free casein 18, salts 4 and corn oil 5, containing 0.02% halibut liver oil and 0.003% α-tocopherol, plus the following vitamins in milligrams per kilogram of ration: thiamine 2.0, riboflavin 3.0, pyridoxine 2.0, nicotinic acid 25, calcium pantothenate 20, 2-methyl-1, 4-naphthoquinone 10, inositol 100, biotin 0.1, folic acid 2.0, choline chloride 1,000, and vitamin B_{12} 0.02. A somewhat similar diet low in pantothenic acid has been described previously (Lih et al., '51).

For the assays of pantothenic acid and in one of the assays for riboflavin all rats were fed the appropriate deficient diet for two weeks, after which the animals were distributed into groups of 5 or 6 and each group fed a different level of the vitamin with or without an antibiotic. There was no preliminary depletion period in the assays for thiamine, nor in the final assay for riboflavin.

RESULTS

Penicillin, streptomycin, and aureomycin all stimulated the growth of rats receiving limiting amounts of thiamine, penicillin being the most effective of the three antibiotics (table 1). In the first series the level of thiamine, 0.5 μg/gm, was so low that it failed to maintain the growth of rats for 4 weeks in the absence of an antibiotic. The rats receiving the antibiotics, however, grew somewhat better than the controls and maintained their weights for a longer period; the rats receiving penicillin continued to grow for 4 weeks, at which time they weighed 115 gm (87 to 139) as compared to only 55 gm (49 to 64) for the control group.

Essentially similar results were observed in a second series in which somewhat higher levels of thiamine were employed (table 1). Growth stimulation was observed with penicillin, streptomycin, and aureomycin, but not with terramycin or

chloromycetin. The quantitative response to penicillin exceeded that observed when the thiamine content of the diet was doubled; after 4 weeks the control rats on 1 mg of thiamine per kilogram weighed 102 gm, and those on 2 mg

TABLE 1

Effects of certain antibiotics on the growth of rats fed limiting amounts of thiamine [1]

SUPPLEMENTS		AVERAGE WEIGHTS AT	
Thiamine	Antibiotic	2 wks.	4 wks.
mg/kg diet	*mg/kg diet*	*gm*	*gm*
	Series I		
..	.....	67	52 (1/5)
..	50 aureomycin	76	56
0.5	.....	78	55
0.5	50 aureomycin	82	76
0.5	50 streptomycin	87	69
0.5	50 penicillin	93	115
1.0	.....	85	79
1.0	50 aureomycin	90	124
	Series II		
..	.....	57	40 (1/5)
..	50 aureomycin	59	50 (4/5)
..	50 penicillin	65	64
1.0	.....	86	102
1.0	50 aureomycin	89	114
1.0	50 streptomycin	90	125
1.0	50 penicillin	97	151
1.0	50 chloromycetin	83	83
1.0	50 terramycin	84	101
1.0	sulfasuxidine (1%)	85	122
2.0	.....	92	143

[1] Average initial weights: Series I 46 gm, series II 41 gm. The numbers in parentheses indicate the number of survivors.

weighed 143 gm, while rats on 1 mg of thiamine plus penicillin averaged 152 gm in weight. The effects due to 1% of sulfasuxidine were variable. Initially there was a noticeable diarrhea, and growth in the presence of the drug was less than in the control group. However, between the second and

4th weeks of the experiment the group receiving the sulfa drug grew much better than the control group or that on aureomycin, and its rate of growth was only exceeded by that of the group receiving penicillin (table 1).

In subsequent series the amounts of penicillin were varied from 5 to 100 mg per kilogram of rations supplying 0.75 or 1 mg of thiamine per kilogram. Growth stimulation was observed on all levels of the antibiotic, and it was only slightly less on the diet containing 5 or 10 mg of penicillin per kilogram than on that containing the highest level. No stimulation was observed when the diet contained 50 mg per kilogram of a penicillin preparation [2] inactivated by penicillinase. The addition of 3-nitro-4-hydroxyphenyl arsonic acid likewise failed to stimulate growth at this level.

In the assay diet for riboflavin, aureomycin and penicillin were both effective in stimulating the growth of rats receiving 0.25 or 0.5 mg of riboflavin per kilogram of ration. The degree of stimulation in two series was at least equivalent to that observed when the vitamin content of the basal diet was doubled. Streptomycin, chloromycetin, and sulfasuxidine were inactive in the presence of the lower level of riboflavin (table 2), but in the presence of 0.5 mg of the vitamin per kilogram of ration some stimulation in growth was noted with streptomycin, chloromycetin and terramycin. Rats fed 3-nitro-4-hydroxyphenyl arsonic acid or sulfasuxidine were slightly, but probably not significantly, heavier than the control group.

Analyses of the livers and blood of rats receiving limiting amounts of riboflavin plus antibiotics showed that the concentrations of this vitamin were very similar in the blood and livers of all groups (table 3), but that the total amounts of riboflavin per liver increased when growth-stimulating antibiotics were fed. Livers from rats on 0.5 mg of riboflavin per kilogram of ration averaged 51.7 μg of riboflavin per liver (range 47.5 to 55) when no antibiotic was fed, while in

[2] Obtained from Mr. Glenn Brewer and Prof. W. H. Peterson of this Department.

the presence of aureomycin or penicillin, the amounts in the liver averaged 78 (70 to 84) and 82 (72 to 99) μg per liver, respectively. Smaller increases were observed in the groups receiving streptomycin or chloromycetin.

Rats receiving limiting amounts of pantothenic acid grew better when aureomycin, streptomycin, or penicillin was fed (table 4) and, as with the other vitamins, the degree of stim-

TABLE 2

Effects of antibiotics on the growth of rats fed limiting amounts of riboflavin [1]

SUPPLEMENTS		AVERAGE WEIGHTS AT	
Riboflavin	Antibiotic	3 wks.	5 wks.
mg/kg diet	*mg/kg diet*	*gm*	*gm*
	Series I		
...	.....	62	65
0.25	.....	65	72
0.25	50 aureomycin	81	94
0.25	50 streptomycin	67	73
0.25	50 penicillin	80	97
0.25	50 chloromycetin	62	64
0.25	sulfasuxidine (1%)	71 (4/5)	76 (4/5)
0.5	.....	74	78
1.0	.....	88	97
1.0	50 aureomycin	106	143
	Series II		
..	.....	65	66
..	50 aureomycin	66	70
..	50 penicillin	70	75
0.5	.....	73	76
0.5	50 aureomycin	96	125
0.5	50 streptomycin	90	105
0.5	50 penicillin	96	118
0.5	50 chloromycetin	79	92
0.5	50 terramycin	71	92
0.5	50 3-nitro-4-hydroxyphenyl arsonic acid	73	85
0.5	sulfasuxidine (1%)	69	81
1.0	.....	93	116

[1] Average initial weights: Series I 55 gm, series II 44 gm. The numbers in parentheses indicate the number of survivors.

ulation in growth was greatest when the amount of vitamin in the diet was relatively low. The results with pantothenic acid, however, differed from those with vitamin B_6 (Linkswiler et al., '51), or with thiamine or riboflavin (tables 1 and 2) in that the antibiotics also stimulated growth when no pantothenic acid was added to the basal ration (table 4).

TABLE 3

Effect of antibiotics on the riboflavin content of liver and of blood plasma

RIBOFLAVIN	ANTIBIOTIC	WEIGHT OF RAT [1]	WEIGHT OF LIVER	RIBOFLAVIN IN LIVER		RIBOFLAVIN IN PLASMA
				Total	Conc.	
μg/kg	μg/kg	gm	gm	μg	μg/gm	μg/ml
..	.....	71	4.0	43	11.1	0.88
..	50 aureomycin	76	4.1	47	12.1	0.86
..	50 penicillin	85	4.2	51	12.9	...
0.5	.....	82	4.6	52	11.4	0.83
0.5	50 aureomycin	135	6.8	78	12.1	1.0
0.5	50 streptomycin	114	5.4	67	12.5	0.78
0.5	50 penicillin	133	7.0	82	11.7	0.87
0.5	50 chloromycetin	102	5.1	60	11.9	...
0.5	50 terramycin	107	4.9	55	11.7	0.71
0.5	50 sulfasuxidine	88	3.9	48	12.3	0.78
0.5	50 3-nitro-4-hydroxy-phenyl arsonic acid	93	4.6	61	13.6	0.92
1	.....	128	6.4	77	12.2	0.82

[1] Average value for three rats.

DISCUSSION

The observation that aureomycin can increase the growth of chicks fed diets low in the animal protein factor (Stokstad et al., '49; Stokstad and Jukes, '49) has been followed by numerous studies on the nutritional effects of antibiotics, with the emphasis largely on vitamin B_{12} and on unknown growth factors. Growth-stimulating effects of antibiotics have usually been observed with crude basal rations, although their effectiveness in certain synthetic diets has also been demonstrated (Moore et al., '46; Whitehill et al., '50). The effect appears to be general: antibiotics stimulate the growth

of rats fed limiting amounts of vitamin B_{12} (Groschke and Evans, '50; Oleson et al., '50; Stern and McGinnis, '50; Stokstad and Jukes, '51), vitamin B_6 (Linkswiler et al., '51), or thiamine, riboflavin or pantothenic acid (tables 1 to 4).

TABLE 4

Effects of antibiotics on the growth of rats fed limiting amounts of pantothenic acid[1]

PANTOTHENIC ACID	ANTIBIOTICS	AVERAGE WEIGHTS AT	
		3 wks.	5 wks.
mg/kg diet	*mg/kg diet*	*gm*	*gm*
	Series I		
..	.....	97	108
8.0	.....	165	223
8.0	50 aureomycin	183	236
16.0	.....	184	234
16.0	50 aureomycin	182	239
	Series II		
..	.....	93	98
..	50 aureomycin	112	130
..	50 streptomycin	111	128
..	50 penicillin	103	127
4.0	.....	126	171
4.0	50 aureomycin	152	213
4.0	50 streptomycin	143	206
4.0	50 penicillin	136	192
8.0	.....	163	220
8.0	50 aureomycin	174	218
48.0	.....	180	229
48.0	50 aureomycin	185	242

[1] Average initial weights after two weeks of depletion: Series I 69 gm, series II 73 gm.

These results are in harmony with the idea that intestinal microorganisms normally absorb limiting nutrients away from the host, and that by suppressing these organisms and thus preventing this "theft," antibiotics enable more of the limiting vitamin to be absorbed by the animal itself. Since different antibiotics affect the various types of microorganisms to different degrees, it is not surprising that their growth-promoting potencies should vary with the vitamin

that happens to be limiting in the basal diet. Thus, penicillin was more effective than the other antibiotics in stimulating growth on low-thiamine diets, suggesting that penicillin is particularly effective in suppressing organisms that absorb this vitamin. By the same token, organisms that absorb pantothenic acid seem to be suppressed most effectively by aureomycin or streptomycin.

At least two other possibilities have been suggested as the mechanism by which antibiotics stimulate growth: they may suppress organisms that are unfavorable because of toxic substances emitted (in other words, the antibiotics cure minor diseases), or they may suppress organisms that limit the availability of some unknown growth factor. While either of these mechanisms may have been operative in the present experiments, we do not believe that they predominated, for then a given antibiotic should have remained superior in its growth-promoting effects on a number of our diets, which differed from one another only in the vitamin that was limiting. Since this did not occur, the simpler explanation is preferred; namely, that antibiotics prevented microorganisms from stealing limiting nutrients from the host. According to this latter view the limiting nutrient need not necessarily be a vitamin, but could be anything that is needed both by the microorganism and the host; e.g., essential amino acids or minerals. Also, the fact that an antibiotic increases growth on any given diet would imply that that diet was nutritionally inadequate. The disturbing effect of antibiotics in the bioassay for vitamin B_6 has already been pointed out (Linkswiler et al., '51). The same considerations apply to bioassays for the other common B vitamins.

SUMMARY

1. Penicillin, aureomycin, and streptomycin stimulated the growth of rats receiving limiting amounts of thiamine, riboflavin or pantothenic acid, as fed in bioassays for these vitamins. The antibiotics were most effective in diets that con-

tained enough vitamin for half-maximum growth, and the growth responses due to the antibiotics were approximately equal to those observed when the vitamin content of the diet was doubled in the absence of antibiotics.

2. The relative effectiveness of the antibiotics varied with the vitamin deficiency. In thiamine deficiency penicillin produced the greatest increases in growth; aureomycin and streptomycin produced definite but lesser increases; while chloromycetin, terramycin and 3-nitro-4-hydroxyphenyl arsonic acid were inactive, and sulfasuxidine exerted a delayed effect. In riboflavin deficiency penicillin and aureomycin were equally effective; some stimulation was noted with streptomycin, chloromycetin and terramycin in the presence of a moderate level of riboflavin; 3-nitro-4-hydroxyphenyl arsonic acid and sulfasuxidine appeared to be inactive. In pantothenic acid deficiency aureomycin and streptomycin were best, while penicillin was less active; the antibiotics also stimulated growth in the absence of added pantothenic acid.

LITERATURE CITED

GROSCHKE, A. C., AND R. J. EVANS 1950 Effect of antibiotics, synthetic vitamins, vitamin B_{12} and APF supplement on chick growth. Poultry Sci., *29:* 616.

LIH, H., T. E. KING, H. HIGGINS, C. A. BAUMANN AND F. M. STRONG 1951 Growth promoting activity of bound pantothenic acid in the rat. J. Nutrition, *44:* 361.

LINKSWILER, H., C. A. BAUMANN AND E. E. SNELL 1951 Effect of aureomycin on the response of rats to various forms of vitamin B_6. Ibid., *43:* 565.

MOORE, P. R., A. EVENSON, T. D. LUCKEY, E. MCCOY, C. A. ELVEHJEM AND E. B. HART 1946 Use of sulfasuxidine, streptothricin and streptomycin in nutritional studies with the chick. J. Biol. Chem., *165:* 437.

OLESON, J. J., B. L. HUTCHINGS AND A. R. WHITEHILL 1950 The effect of feeding aureomycin on the vitamin B_{12} requirement of the chick. Arch. Biochem., *29:* 334.

STERN, J. R., AND J. MCGINNIS 1950 Antibiotics and early growth of rats fed a soybean oil meal diet. Ibid., *28:* 364.

STOKSTAD, E. L. R., AND T. H. JUKES 1949 Further observations on the animal protein factor. Proc. Soc. Exp. Biol. Med., *73:* 523.

———— 1951 Effect of various levels of vitamin B_{12} upon growth response produced by aureomycin in chicks. Ibid., *76:* 73.

STOKSTAD, E. L. R., T. H. JUKES, J. PIERCE, A. C. PAGE, JR. AND A. L. FRANKLIN 1949 The multiple nature of the animal protein factor J. Biol. Chem., *180:* 647.

WHITEHILL, A. R., J. J. OLESON AND B. L. HUTCHINGS 1950 Stimulatory effect of aureomycin on the growth of chicks. Proc. Soc. Exp. Biol. Med., *74:* 11.

EFFECT OF DIET ON MAZE LEARNING IN SECOND-GENERATION RATS. FOLIC ACID DEFICIENCY [1]

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Dietary dificiencies of many of the B vitamins are known to result in the degeneration of nerve tissue or the impairment of its function. To cite only a few examples, Prickett ('34) studied thiamine deficiency in the mouse; Phillips and Engel ('38), riboflavin deficiency in the chicken; Street et al. ('41), pyridoxine deficiency in the dog; and Lippincott and Morris ('41), pantothenic acid deficiency in the mouse. The effect of thiamine deficiency on learning in children has been studied by Harrell ('46) and Robertson et al. ('47) and its effect on maze learning in the rat by O'Neill ('49).

Windle et al. ('43, '44) have studied the effects in guinea pigs of asphyxiation *in utero*. All the animals showed symptoms of a neurological nature after birth and marked structural changes in the brain. The learning ability of these animals was studied in a simple alternation type maze, and it was found that a large percentage of the asphyxiated animals which outwardly appearad normal were inferior in learning the maze problem.

O'Dell, Whitley and Hogan ('48) found that folic acid has a beneficial effect in the prevention of hydrocephalus, a congenital abnormality of the central nervous system, when

[1] Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series No. 1254. This investigation was supported in part by a research grant from the National Institutes of Health, U. S. Public Health Service.

added to a synthetic diet similar to the one used by Richardson and Hogan ('46) to produce hydrocephalus. This diet contained casein as the source of protein and all the vitamins required by the rat for growth, but about 2% of the offspring of the animals fed this ration were hydrocephalic. When the dams received liver extract in the diet, all the offspring were normal. Gross symptoms of the abnormality included an enlarged cranium and loss of equilibrium, and the defect could be detected in newborn animals by the increased light transmission through the cranium. The technique readily detected overt hydrocephalus, but it seemed likely that there were cases of damage to the brain which this method would not detect. Maze learning was chosen as a technique for studying the psychological behavior of offspring which showed no gross symptoms of hydrocephalus, but which were produced by dams that received the deficient diet.

The present report describes the results obtained in maze studies on animals from dams fed a synthetic diet and reared to maturity on the same diet, as compared to control animals from the stock colony. The effect of folic acid on maze learning when it is added as a supplement to the basal diet is also reported.

EXPERIMENTAL

The female rats used in this experiment were transferred at maturity from the stock diet to the experimental rations. The normal-appearing offspring were weaned at 4 weeks of age and fed the experimental rations until they were used for maze studies at 8 to 10 weeks of age. At that time all animals were transferred to the stock ration (1428) and the experimental animals selected for maze trials were paired with controls from the stock colony of the same age, sex and weight (within 10 gm). The average weights of the animals varied from 125 to 175 gm. The composition of the rations is shown in table 1.

The maze used was a modified Stone multiple-T water maze containing 14 choice points. A diagram and detailed

description of the maze and its operation have been presented by Marx ('48). The motive for negotiating the maze was escape from water. The runways were filled with water to a depth of 8 inches and the temperature was maintained at about 20°C. After each trial the animals were allowed to become well dried and rested in a heated turntable. The interval between trials varied with the time spent in the

TABLE 1

Compositions of rations

CONSTITUENT	RATION NUMBER		
	1428	1953	1956
	gm	*gm*	*gm*
Yellow corn	48.5		
Soybean oil meal	20.0		
Alfalfa leaf meal	3.0		
Fish meal	15.0		
Brewers' yeast	10.0		
Steamed bone meal	1.0		
Sodium chloride	0.5		
Cod liver oil	2.0		
Water-washed casein		30.0	30.0
Cerelose		52.0	52.0
Wood pulp		3.0	3.0
Salts [1]		5.0	5.0
Lard		10.0	10.0
Total	*100.0*	*100.0*	*100.0*
Supplements			
Vitamin mixture lacking pteroylglutamic acid [2]	0.0	see [2]	see [2]
Pteroylglutamic acid [3]	0.0	0.0	0.5 mg

[1] The salt mixture used was that described by Richardson and Hogan ('46).

[2] The vitamin supplement supplied the following substances in the amounts indicated per 100 gm of the ration: vitamin A, 2,000 I.U.; vitamin D, 285 I.U.; alpha-tocopherol, 2.5 mg; menadione, 1.0 mg; thiamine hydrochloride, 1.6 mg; riboflavin, 1.6 mg; pyridoxine hydrochloride, 1.6 mg; calcium pantothenate, 4.0 mg; niacin, 5.0 mg; biotin, 0.02 mg; and choline chloride, 100 mg. The crystalline vitamins were supplied through the courtesy of Dr. D. F. Green, Merck and Company, Rahway, New Jersey.

[3] The pteroylglutamic acid was supplied through the courtesy of Dr. T. H. Jukes, the Lederle Laboratories, Pearl River, New York.

water and ranged from two hours in the first trial to a minimum of 20 minutes in later trials. The operator of the maze was unaware of the group to which each animal belonged, thus eliminating the possibility of his subjectively influencing the results. The rats were given 5 trails daily for 12 days, or a total of 60 trials. The criterion of mastery chosen for evaluating the results was the completion of one trial without error, and the results are expressed as the number of trials required to learn the maze and as the number of errors made before learning. Calculations made on the basis of 4 perfect trials out of 5 gave approximately the same degree of significance. The average number of trials and number of errors are expressed as the nearest whole number.

The results obtained are presented in table 2. Twelve groups of animals containing a total of 78 pairs were given maze trials over a period of 4 years, and in each group the average number of trials required by the rats fed the basal synthetic ration (1953) was greater than that required by the control animals fed the stock ration (1428). To master the maze, the 78 control animals required an average of 14 trials and made an average of 186 errors per rat, whereas the rats fed the basal ration (1953) required 22 trials and made 288 errors per rat. Fischer's "t" test for reliability of differences between means was employed, and the probabilities of the occurrence of reliable differences were obtained from Fischer's table of P values. When ration 1428 is compared with ration 1953 on either the basis of number of trials required to learn the maze or on number of errors made, the probability of the difference occurring due to chance alone is less than 0.01. This is highly significant evidence that a deficiency of a nutritional factor in the maternal diet results in inferior maze learning ability in offspring which are reared to near maturity on the same ration.

After O'Dell et al. ('48) observed that folic acid decreased the incidence of hydrocephalus, offspring from dams fed the folic acid-supplemented ration (1956) were given maze trials, using the stock ration for positive controls and ration 1953

TABLE 2

Relation of diet to maze learning [1]

GROUP	DATE STARTED	NO. OF PAIRS	RATION 1428 (STOCK)		RATION 1953 (BASAL)		RATION 1956 (FOLIC ACID SUP'T.)	
			Ave. number		Ave. number		Ave. number	
			Trials	Errors	Trials	Errors	Trials	Errors
1	4–24–46	8	10	168	15	223		
2	5–2–46	8	11	143	17	218		
3	6–18–46	8	16	225	31	398		
4	6–24–46	5	11	168	20	276		
5	1–7–46	11	18	234	25	314		
6	3–18–47	7	20	221	27	381		
7	10–27–48	4	14	183	19	239	14	240
8	12–15–48	4	12	183	20	281	15	243
9	1–17–49	6	11	127	18	298		
10	3–14–49	5	11	162	15	223	12	185
11	3–28–49	5	13	217	20	253	13	206
12	8–16–50	7	15	157	29	292		
Final average		78	14	186	22	288		
Final average, groups 7, 8, 10, 11		18	12	187	18	248	13	216

[1] A statistical analysis of the data in this table shows that there was a significant difference between rations 1428 and 1953 ($P < 0.01$) and that there was no significant difference between rations 1428 and 1956.

for negative controls. Groups 7, 8, 10 and 11 (table 2) contained a total of 18 animals from each ration. In order to learn the maze the positive controls required an average of 12 trials and made on the average 187 errors per rat; the negative controls required 18 trials and made 248 errors; and the folic acid-supplemented group required 13 trials and made 216 errors. A statistical analysis of these data shows no difference between the folic acid-supplemented animals and the stock animals, but there was a significant difference between those fed the basal and those fed the stock rations.

DISCUSSION

These results show that rats born to dams fed a ration low in folic acid (microbiological assay indicates that the basal ration contained about 5 μg of folic acid per 100 gm) produce offspring which if fed this same ration until near maturity show inferior maze learning ability. Although our data at present are insufficient to permit the drawing of a definite conclusion, it seems highly probably that the damage to the brain occurs before birth and that feeding the basal ration subsequently has little if any effect on maze learning. This hypothesis is supported by the fact that a small percentage of the offspring from dams on the same ration were hydrocephalic. Overholser et al. ('50) studied the brains of hydrocephalic animals and observed that lateral ventricles were greatly distended and that the cerebral aqueduct was round in the anterior portion, rather than having its normal irregular shape. Unpublished observations show that some normal-appearing litter mates of hydrocephalic animals show an abnormal cerebral aqueduct. Examination of brains from embryos indicates that hydrocephalus is caused by closure of the cerebral aqueduct at about the 18th day (Overholser et al., '51). There appears to be a higher percentage of occluded aqueducts at this stage than among newborn animals, indicating that in some cases the aqueduct opens at, or shortly before, birth. It is possible that a temporary closure of the cerebral aqueduct in animals which do

not show gross symptoms of hydrocephalus may result in increased ventricular pressure during the development of the cortex and cause permanent damage to the tissue.

The maze studies reported here are unique in at least two respects. As far as the authors are aware, this is the first study which suggests a functional deterioration in the central nervous system as a result of inadequate maternal nutrition. It should also be pointed out that in most maze experiments designed to study the effect of a nutritional deficiency it is very difficult to select animals of the same age and weight because of the effect of the deficiency on growth. A difference in weight and vigor may have a marked effect on ability to negotiate a maze. Since supplementation of the diet with folic acid is not required for the growth of the rat, animals on the basal and folic acid-supplemented ration grew at approximately the same rate and both groups were normal in appearance. The fact that the animals could be readily paired tends to rule out factors other than the innate ability to learn.

SUMMARY

Rats born to dams fed a ration low in folic acid and reared to near maturity on the same ration showed inferior maze learning ability as compared to paired controls from the stock colony. Supplementation of the maternal diet with folic acid resulted in offspring which learned as readily as the control animals.

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LITERATURE CITED

HARRELL, RUTH F. 1946 Mental response to added thiamine. J. Nutrition, *31:* 283.

LIPPINCOTT, S. W., AND H. P. MORRIS 1941 Morphologic changes associated with pantothenic acid deficiency in the mouse. J. Natl. Cancer Inst., *2:* 39.

MARX, MELVIN H. 1948 Effects of supranormal glutamic acid on maze learning. J. Comp. Physiol. Psychol., *41:* 82.

O'DELL, B. L., J. R. WHITLEY AND A. G. HOGAN 1948 Relation of folic acid and vitamin A to incidence of hydrocephalus in infant rats. Proc. Soc. Exp. Biol. Med., *69:* 272.

O'NEILL, PHILIP H. 1949 The effect on subsequent maze learning ability of graded amounts of vitamin B_1 in the diet of very young rats. J. Genetic Psychol., *74:* 85.

OVERHOLSER, M. D., J. R. WHITLEY, B. L. O'DELL AND A. G. HOGAN 1950 A study of hydrocephalic brains of rats from dams on a vitamin deficient diet. Anat. Rec., *106:* 62.

———— 1951 A study of hydrocephalic brains of prenatal and postnatal rats from dams on a diet deficient in vitamin B_{12}. Ibid., *109:* 73.

PHILLIPS, P. H., AND R. W. ENGEL 1938 Histopathology of neuromalacia and "curled-toe" paralysis in the chick fed low riboflavin diets. J. Nutrition, *16:* 451.

PRICKETT, C. O. 1934 The effect of a deficiency of vitamin B_1 upon the central nervous systems of the rat. Am. J. Physiol., *107:* 459.

RICHARDSON, L. R., AND A. G. HOGAN 1946 Diet of the mother and hydrocephalus in infant rats. J. Nutrition, *32:* 459.

ROBERTSON, E. C., C. M. TATHAM, N. F. WALKER AND M. R. WEAVER 1947 The effect of thiamine on growth, vision and learning, using identical twins. Ibid., *34:* 691.

STREET, H. R., G. R. COWGILL AND H. M. ZIMMERMAN 1941 Vitamin B_6 deficiency in the dog. Ibid., *21:* 275.

WINDLE, W. F., AND R. F. BECKER 1943 Asphyxia neonatorum. An experimental study in the guinea pig. Am. J. Obst., *45:* 183.

WINDLE, W. F., R. F. BECKER AND A. WEIL 1944 Alteration in brain structure after asphyxiation at birth. An experimental study in the guinea pig. J. Neuropath. Exp. Neurol., *3:* 224.

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1952 Award of $1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Jury of Award of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1952.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Jury of Award, nominations for this award for work published in 1951 must be in the hands of the Chairman of the Nominating Committee by January 1, 1952. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Jury of Award in its consideration of the nomination.

SAMUEL LEPKOVSKY
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CHAIRMAN, NOMINATING COMMITTEE

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1952 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1952.

HENRY A. MATTILL
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CHAIRMAN, NOMINATING COMMITTEE

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1952 Award of $1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1952. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1952. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

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